POLYMORPHISMS AND BIOCHEMICAL PROPERTIES OF PEROXIDASE ISOZYMES IN MAIZE (*ZEA MAYS* L.)

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
INTRODUCTION	1
LITERATURE REVIEW	3
Epigenetic Variations of Peroxidase Isozymes	3
Thermal Sensitivity of Peroxidases	4
Polyphenoloxidase and Laccase	6
Ontogeny, Tissue-specificity and Intracellular Localization of Peroxidases	9
1) Ontogeny	9
2) Tissue Specificity of Peroxidases	10
3) Histochemical Localization of Peroxidases	10
4) Extraction of Wall-bound Peroxidases	11
5) Association of Peroxidases with Cell Particulates	12
Regulation of Peroxidase Activity by Plant Growth Hormones	13
IAA oxidase activity of Peroxidases	16
Peroxidase and Lignification	19
Effects of Ionizing Radiations on Peroxidases	21
GENERAL MATERIALS AND METHODS	24
Materials	24
Methods	24
RESULTS AND DISCUSSION	31
I. INTRACELLULAR LOCALIZATION OF PEROXIDASES	31

TABLE OF CONTENTS (Contd.)

		Page
	Extraction and Separation of Cytoplasmic and Cell Wall-bound Peroxidases	31
	Effect of Calcium Ion Concentration and Extraction Time on Peroxidase Release from Cell Wall Fraction	33
	Ca^{++}-soluble versus Ca^{++}-insoluble Peroxidases in Walls .	33
	Physiological Significance of Peroxidases in Cytoplasmic and Wall-bound Fractions	39
II.	ONTOGENETIC VARIATIONS OF PEROXIDASE ISOZYMES	48
III.	THERMAL SENSITIVITY, SUBSTRATE SPECIFICITY AND pH RESPONSE OF PEROXIDASE ISOZYMES	56
	Thermal Sensitivity of Peroxidase Isozymes	56
	Substrate Specificity of Peroxidases	59
	pH Response of Peroxidases	63
IV.	IAA OXIDASE ACTIVITY OF MAIZE PEROXIDASES	67
	Peroxidase and IAA oxidase Activities of Leaf Extract . .	67
	Effect of Substrate and Enzyme Concentrations	68
	Effect of pH on IAA Oxidase Activity	72
	Effect of Manganese on IAA Oxidase Activity	74
	Effects of Monophenols and Hydrogen Peroxide on IAA Oxidase Activity	76
	IAA Oxidase Activity on Gels of Maize Peroxidases	79
	Relationship Between Peroxidases and IAA Oxidases	84
V.	EFFECT OF IONIZING RADIATION ON PEROXIDASE AND IAA OXIDASES	87
	Effects of Gamma-irradiation on Plant Growth and Total Oxidase Activities	87
	Effects of Ionizing Radiation on Isozymes of Peroxidase and IAA Oxidase	91
	The Mechamism of Radiation Activation of Peroxidases and IAA Oxidases	93

TABLE OF CONTENTS (Contd.)

Page

VI. REGULATION OF PEROXIDASE ACTIVITY BY AN INHIBITORY SUBSTANCE FROM MAIZE POLLEN GRAINS 94

Detection of the Inhibitor 94

Isolation and Partial Purification of the Inhibitor 94

Effect of pH on the Inhibitory Action 97

Effect of the Inhibitor Concentration 97

Effect of Preincubation Time on Inhibition 100

Thermal Stability of the Inhibitor 100

Specificity of the Inhibitor 100

Chemical Properties of the Inhibitor 102

Mode of Action of the Inhibitor 106

Future Studies of the Nature of the Inhibitor 107

SUMMARY 108

LITERATURE CITED 111

LIST OF TABLES

Table		Page
1	Tissue polymorphisms of the twelve maize peroxidases	30
2	Amounts of Ca^{++}-soluble and Ca^{++}-insoluble peroxidases in walls of 15 day-old maize tissues	38
3	Amounts of twelve peroxidase enzymes in cytoplasmic (C) and wall (W) fractions of maize tissues	43
4	Amounts of cytoplasmic (C) and wall-bound (W) peroxidases in leaves of brown midrib-3 (bm_3) and brittle stalk-2 (bk_2)	46
5	Substrate specificity of twelve peroxidases in maize	61
6	Peroxidase activity of crude extract of maize leaves	68
7	Effects of $MnCl_2$ (10^{-4} M) and TCP (10^{-6} M) on maize leaf IAA oxidase activity	78
8	Effects of hydrogen peroxide and cofactors on the maize leaf IAA oxidase activity	78
9	Effect of preincubation time of peroxidase with the inhibitor on peroxidase inactivation	101
10	Thermal stability of the inhibitor	101
11	Effect of the inhibitor on peroxidase activity from different maize tissues	101

LIST OF FIGURES

Figure		Page
1	Approximate positions on 7% acrylamide gels at pH 8.1 of the major allelic isozymes of the 12 maize peroxidases	29
2	Flow chart of peroxidase extraction from cytoplasmic and wall-bound fractions of maize tissues	31
3	Effect of calcium ion on peroxidase release from isolated cell wall of 15 day-old maize leaves	34
4	Effect of time of incubation with isolated cell wall of 15 day-old leaves and 0.2 M $CaCl_2$ on peroxidase release	35
5	Zymogram of peroxidase isozymes in cytoplasmic (CYTO) and wall-bound (WALL) fractions of 7 day-old coleoptiles	36
6	Zymogram of peroxidase isozymes in cytoplasmic (CYTO) and wall-bound (WALL) fractions of 15 day-old whole roots	40
7	Densitometric tracings of peroxidase isozymes in cytoplasmic and wall-bound fractions of whole roots	41
8	Changes of soluble peroxidase activity during growth of leaves (●—●), mesocotyls (o—o) and roots (▲—▲)	49
9	Zymogram of cytoplasmic peroxidase isozymes in the developing mesocotyls from 5 to 9 day-old seedlings	50
10	Densitometric tracing of peroxidase isozymes in Figure 9	51
11	Zymogram of cytoplasmic (C) and wall (W) peroxidase isozymes in the developing mesocotyls from 5 to 9 day-old seedlings	54
12	Regeneration of thermally inactivated peroxidase in mixtures of maize leaves and coleoptiles of 7 day-old seedlings	57
13	Regeneration of thermally inactivated peroxidase isozymes of maize leaves and coleoptiles from 7 day-old seedlings	58

LIST OF FIGURES (Contd.)

Figure		Page
14	pH optima of peroxidase isozymes in extract of 15 day-old maize mesocotyls	64
15	Effect of pH on peroxidase activity of maize mesocotyls of 15 day-old seedlings	66
16	Rate of IAA destruction at different pH in the presence and absence of 10^{-4} M $MnCl_2$	69
17	Rate of enzymatic destruction of IAA as a function of substrate concentration	70
18	Rate of enzymatic inactivation of IAA as a function of enzyme concentration	71
19	Effect of pH on IAA oxidase activity of maize leaf extract	73
20	Effect of $MnCl_2$ on IAA oxidase activity of maize leaf extract	75
21	Zymograms of peroxidase (L) and IAA oxidase (2) in leaf extract of 15 day-old seedlings	81
22	Zymograms of peroxidase (1) and IAA oxidase stained with (2) and without (3) 0.0006% H_2O_2 after electrophoresis of 15 day-old maize leaf extract	83
23	Zymograms of peroxidase (1) and IAA oxidase stained with 0.0006% (2) and 0.6% (3) H_2O_2 after electrophoresis of maize leaf extract	83
24	Growth rates of mesocotyls of gamma-irradiated corn seeds between 0 and 500 krads	88
25	Effect of gamma-irradiation with Cobalt-60 at dose levels of 0 to 500 krads on the total peroxidase activity of mesocotyls	89
26	Effect of gamma-irradiation with Cobalt-60 at dose levels of the total IAA oxidase activity of mesocotyls	90
27	Zymograms of peroxidase and IAA oxidase of roots irradiated with gamma-rays between o and 500 krads	92

LIST OF FIGURES (Contd.)

Figure		Page
28	Oxidation of guaiacol by pollen peroxidase.	95
29	Oxidation of o-dianisidine by pollen peroxidase	96
30	Activity of pollen peroxidase with (o—o) or without (●—●) the inhibitor at different pH values	98
31	Inhibition of pollen peroxidase activity as a function of amounts of the inhibitor	99
32	Activity of catecholase from maize leaf with (o—o) or without (●—●) the inhibitor	103
33	The ultraviolet spectrum of partially purified inhibitor from pollen grains in ethanol solution	105

INTRODUCTION

Peroxidases have been investigated in various plant tissues and found to be normal components of such tissues. The wide occurrence, highly polymorphic nature and broad substrate specificity of peroxidases have stimulated research into their physiological and biochemical properties.

Several types of physiological roles have been proposed for peroxidases in plants. Biochemical and physiological studies of isolated peroxidases and of mutant plants suggest that peroxidases are involved in the regulation of plant growth substances (Kamerbeek 1956; McCune 1961) and in the synthesis of cell wall components such as lignin (Kuc and Nelson 1964; Higuchi 1971; Sarkanen and Ludwig 1971).

Other functions of plant peroxidases are suggested by their broad substrate specificity (Rychter and Lewak 1971; Brewbaker and Hasegawa 1974a), high tissue specificity (Scandalios 1964; Gordon and Alldridge 1971) and ontogenetic variations (Hamill and Brewbaker 1969; Lodha et al. 1974). However, methods used to determine peroxidase distribution in plant tissues containing several isozymes are not accurate below the tissue level. Most plant tissues are not homogenous, and such assay methods usually involve analysis of tissues containing more than one cell type.

Many enzymes are compartmentalized within plant cells, associated especially with the cell wall, mitochondria and plastids (Christie and Judah 1954; Goddard and Stafford 1954; Lanzani and Galante 1964; Haard 1973). These differences in localization of isozymes permit them to have distinct biochemical and charge properties, and physiological roles.

Maize (Zea mays L.) genetic studies have revealed the genetic basis for isozyme variations of nine peroxidases, among the twelve major peroxidases of this species (Brewbaker and Hamill 1972). The principal objectives of the present study was to elucidate the physiological roles of the twelve maize peroxidases, through analysis of their tissue and substrate specificity, their intracellular localization, and their biochemical and physicochemical properties.

LITERATURE REVIEW

Peroxidase (donor: H_2O_2 oxidoreductase; E.C. 1.11.1.7) is ubiquitous among higher plants, and regularly exists in multiple forms or isozymes. Peroxidase catalyzes the oxidation of organic compounds (hydrogen donors) in the presence of organic peroxide (hydrogen acceptor). The enzyme is highly specific for the hydrogen acceptor--only hydrogen peroxide (HOOH), methyl hydrogen peroxide (CH_3OOH) and ethyl hydrogen peroxide (C_2H_5OOH) are active (George 1953a, 1953b, Paul 1963). In contrast with this, a large number of hydrogen donors are known, including phenols, diamines, indophenols, leucodyes, ascorbate and certain acids (Mason 1957). Thus, peroxidase is known to have broad "substrate" specificity. However, peroxidase is rather high specific in regard to organic peroxide as a substrate. The peroxide is first combined with enzyme molecule (complex I) and then the complex I oxidizes the hydrogen donor. This is represented as a general formula below (modified from Barman, 1969);

$$\underset{\text{(Peroxidase)}}{Px\cdot Fe^{3+}(H_2O)} + H_2O_2 \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \underset{\text{(Complex I)}}{Px.Fe.H_2O_2} + H_2O$$

$$\text{Complex I} + \underset{\text{(Organic compound)}}{AH_2} \xrightarrow{k_7} \underset{\text{(Complex II)}}{Px.Fe(OH\cdot)} + AH. + H_2O$$

$$\text{Complex II} + AH \xrightarrow{k_4} \underset{\text{(Peroxidase)}}{Px.Fe^{3+}(H\ O)} + AH\cdot$$

Complex I has a very broad substrate specificity and is usually considered a peroxidase.

Epigenetic Variations of Peroxidase Isozymes

The zymogram technique provides an easy means of recognizing the

multiple molecular forms of peroxidase in plant tissues. However, isozymes may be generated as artifacts from preexisting forms of the enzyme either in vivo during development or in vitro during extraction and purification (Shapiro and Parker 1960; Margoliash and Lustgarten 1961; Bingham et al. 1964; Staples et al. 1965; Delincee 1971; Macnicol 1973). Liu and lamport (1973) reported that new isozymes were generated by treatment of horseradish peroxidase (HRP) by incubation with varied pH solution. Isozyme distribution patterns were altered, but there was no change in total peroxidase activity present in the extract. It seems that relative mobility of isozymes were changed by the change of pH values, resulting in apparent new isozymes.

The term "metazyme" has recently been applied to epigenetic variants in serum and urine hydrolases (Ogita 1974), while the term "pseudoisozyme" has been applied in the study of phosphoglucose isomerases by Noltmann (1974) and of hepatic tyrosine aminotransferase by Gelehrter and Spencer (1974). Ascertaining that variations are truly epigenetic should involve several experimental methods as exemplified by the study of Liu and Lamport (1973).

Thermal Sensitivity of Peroxidases

The regeneration of thermally-inactivated peroxidase has been extensively studied since 1901, and reviewed by Schwimmer (1944). Peroxidase is one of the most heat resistant enzymes of plant tissues, and can cause the production of off-flavors during food processing. Accordingly, peroxidase is frequently used in industry as a test substance to determine whether or not vegetables have been heated sufficiently in blanching (Kiermeier and Herrlinger 1951; Morris 1958; Mundt and McCarty 1960).

The mechanism of heat regeneration of peroxidase has been the subject of many studies. Gallagher (1924) reported that the mango peroxidase contained iron and also gave several reactions for aldehyde. He suggested that the aldehyde was a zymogen or precursor of the heated enzyme and the enzyme was regenerated by catalytic action of iron. Studies of Bach and Wilensky (1930) showed that red radish peroxidase extracts freed from non-colloidal components by ultrafiltration were more heat resistant than the original extracts. At boiling temperature both the colloidal ultrafiltration residue and the original extract were equally inactivated, but the former regenerated its ability more quickly. Regeneration is also influenced by the reaction medium of the enzyme. For example, Pronin (1931) reported that the optimum pH for the regeneration of heat-inactivated radish peroxidase was at neutrality. In another study, Schwimmer (1944) showed that the regeneration of cabbage or turnip peroxidases required both the precipitate formed upon heating and the supernatant.

These earlier studies on peroxidase regeneration usually assumed that only one enzyme was responsible for the reaction under observation. According to kinetic studies of thermal inactivation and regeneration of peroxidase by Yamamoto et al. (1962), peroxidase in whole kernels of corn consisted of both heat-labile and heat-stable fractions. They suggested the possibility of multiple enzyme groups having different heat-sensitivity. Chenchin and Yamamoto (1973) found one extremely heat-labile isozyme in pericarp, germ and endosperm of corn. This isozyme (A-5) corresponds to peroxidase 9 (Figure 1) and its heat-lability was also confirmed (Figure 13). They also assumed that most of isozymes consisted of a heat-labile and a heat-stable portion within a molecule.

For example, isozyme C-2 in pericarp of sh2 SYN 2e was much more heat-stable than C-2 in pericarp of H68. The former C-2 was assumed to contain more heat resistant portion than the latter C-2. They heated different fresh tissues of different varieties and then extracted the enzyme with 0.1 M NaCl in phosphate buffer. Even the same isozyme might have different heat-stability if it was heated in different media (tissue or variety). Their extraction method, however, did not allow the sufficient release of wall-bound peroxidases (Haard 1973).

Polyphenoloxidase and Laccase

In addition to peroxidase, other enzymes are also known to use phenolic compounds as a substrate. In its natural state, peroxidase is often found accompanied by these phenol oxidases which may influence or modify peroxidase action. Usually these phenol oxidases are divided into two types; the tyrosinase type and the laccase type.

The tyrosinase is a copper protein and also has the trivial names o-diphenol oxidase, polyphenoloxidase, phenolase, phenol oxidase, catecholase or catechol oxidase. The systematic name and numbering of this enzyme are; o-diphenol: oxygen oxidoreductase, E.C. 1.10.3.1 (Barman 1969). It catalyzes the following generalized reaction;

$$2 \text{ o-diphenol} + O_2 = 2 \text{ o-quinone} + 2H_2O$$

Laccase, also a copper protein, was first isolated from the latex of the Japanese Lack tree, Rhus vernicifera (Yoshida 1883), the source of commercial lacquer. Its systematic name and numbering are; p-diphenol: oxygen oxidoreductase, E.C. 1.10.3, and its catalyzes the following generalized reaction (Barman 1969);

$$2 \text{ p-diphenol} + O_2 = 2 \text{ p-quinone} + 2H_2O$$

Tyrosinase and laccase might be separated on the basis of their activity on certain substances as shown below;

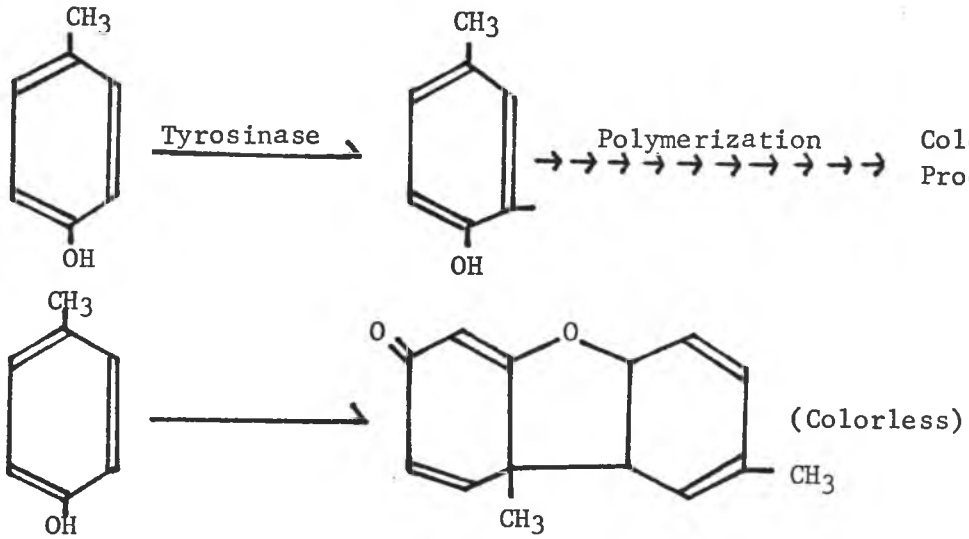
	Tyrosine	p-Cresol	p-phenylenediamine	Hydroquinone
Tyrosinase	+	+	-	-
Laccase	-	-	+	+

Although this substrate approach has been used widely to distinguish between the two enzymes (Gregory and Bendall 1966; Rychter and Lewak 1961), it is not entirely satisfactory for several reasons. First, in addition to p-dihydric phenols, laccase is also reported to attack o-dihydric phenols as tyrosinase does (Fahraeus and Ljunggren 1961a, 1961b). Secondly, it has been reported that the laccase also attacks p-cresol (Fahraeus and Ljunggren 1961a, 1961b).

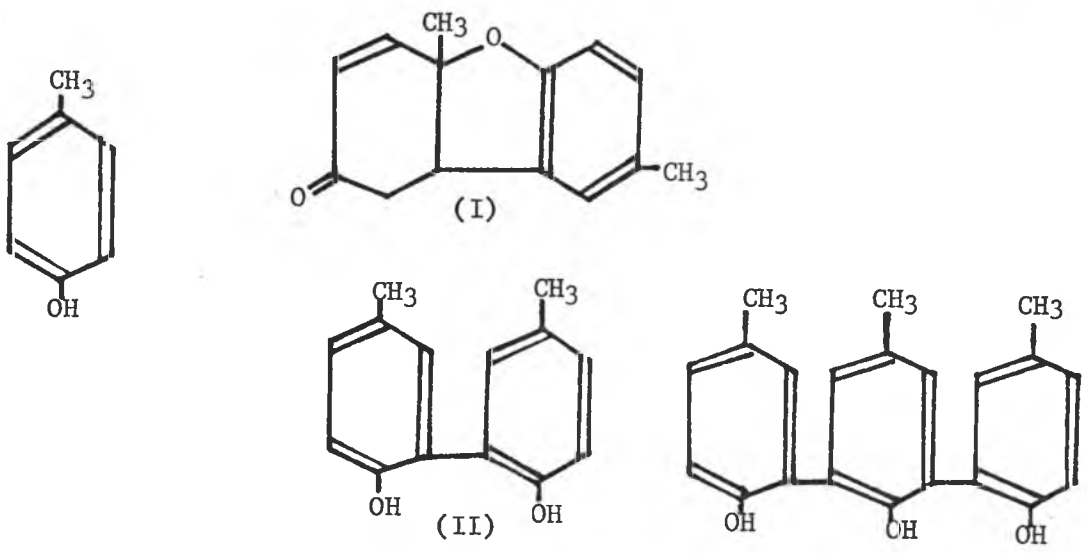
Accordingly, it is reasonable to distinguish between these two enzymes by their physical and chemical characteristics or by differences of their reaction products. For example, laccase has been reported to have greater stability toward heat and alcohol treatment (Dawson and Tarpley 1951). Other characteristics are listed below (Brown 1967).

Property	Tyrosinase	Laccase
State of copper	Cu^{+}(?)	Cu^{++}
UV absorption, nm	280	280, 615
Inhibition by CO	+	-

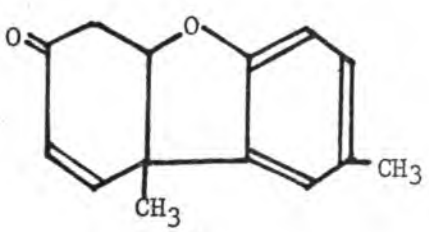
Also, the product of p-cresol by the action of tyrosinase and laccase has been reported to be different as follows: (Fahraeus and Ljunggren 1961a, 1961b);



In relation to these two phenol oxidases, the oxidized products of p-cresol by peroxidase in the presence of hydrogen peroxide are as followed (Westerfelt and Lowe 1942);



Compound I was thought to be the main product, but Barton et al. (1956) considered the formula shown below to be a more possible representation of compound I.



Ontogeny, Tissue-specificity and Intracellular Localization of Peroxidases

1) Ontogeny

Variations of peroxidases have been reported at different stages in many plants. Ontogenetic variations of peroxidases may be closely related to metabolic events at different stages of development. Haskins (1955) demonstrated the increase of total peroxidase activity, along with enzymes such as catalase, cytochrome oxidase, phosphatase and polyphenoloxidase, during the development of etiolated shoots of corn. A similar but less pronounced effect of age was also observed in green seedlings. Plants grown in the dark differed greatly from normal plants exposed to light. Such plants have etiolated stems, poor development of leaves, less pigmentation or lack of certain internal structural differentiation. At least some of these characteristics can result from high ethylene production (Abeles 1972, 1973). Peroxidase was reported to be involved in ethylene production (Milborrow 1973). Higher peroxidase activity in etiolated plant might be related to the regulation of ethylene.

Changes of total peroxidase activity during growth and senescence are common observations (Bailey and McHargue 1943; Jensen 1955; Alvarez 1968, Hamill and Brewbaker 1969), and these changes were accompanied by changes of specific isozymes. According to Lodha et al. (1974), the peroxidase isozyme patterns were similar in normal and opaque-2 endosperm at 15, 20 and 25 days post-pollination. However, in normal endosperm, a band at Rp (Relative position, same as Rf) 0.14 was absent at 15 and 20 days and another band at Rp 0.21 was absent at 20 and 25 days. In another study, Gordon (1971) observed a major peroxidase isozyme band with the development of photosynthetic activity (leaf development) in the expanding leaf zone of eastern cottonwood. There is no direct evidence

available concerning the involvement of peroxidases in photosynthetic system. However, this has been substantiated by general studies (macnicol 1966; Brewbaker and Hasegawa 1974) but critical studies are lacking. Even though some peroxidase were related to the development of photosynthesis, these isozymes are probably not involved in the photosynthetic processes. These isozymes might be working to detoxify the inhibitors of the photosynthesis such as some phenolic compounds.

2) Tissue Specificity of Peroxidases

Tissue polymorphisms of peroxidase isozymes are extensive in all species studied, occasionally with specific tissue-isozyme associations implying functional roles of different isozymes. According to Macnicol's report (1966), among pea peroxidase isozymes, specific peroxidases C_1 and C_2 were associated with leafy tissues and not detected in roots. A similar result was also obtained by Dvorak and Cernohorska (1967) in pumpkin plants, where two peroxidase zones C_1 and C_2 were clearly detected in leaves but less evident with roots. Zone D was characteristic only for roots. These tissue-specific isozymes might be involved in the specific functional phase of the tissue. The broad substrate specificity of peroxidase (complex I ?) might be related to its high tissue specificity. However, peroxidase is not the only enzyme of high tissue specificity. Several enzymes were reported to be very specific among tissues (Scandalios 1964; Upadhya and Yee 1968).

3) Histochemical Localization of Peroxidases

Hematin peroxidase was one of the first enzymes to be histochemically detected in certain tissues (Van Fleet 1947). The pattern of peroxidase distribution was studied in several representative vascular plants. The

peroxidase system appeared to be correlated with differentiation, though little was mentioned on how the correlation became established and what its nature was. Van Fleet (1959) also observed that peroxidase was detectable in all tissues of several unrelated plants such as the developing ovaries and embryo sacs of corn, the wound meristems of coleus, sansevieria, Cucurbita maxima and Smilax hispida, root tips of Allium sativum. Peroxidase was detectable in advance of or accompanying cell division and declined after the division phase. Decline of peroxidase at the end of the division phase was related to the increase of phenols, naphthols and polyphenoloxidase. According to De Jong (1967), peroxidase localization was cytoplasmic in juvenile onion root tissues and associated with the walls in mature cells. Similarly, histochemical studies by Raa (1973) suggested that peroxidase was normally associated with cell walls during later stages of cell differentiation.

4) Extraction of Wall-bound Peroxidases

In addition to histomchemical methods the intracellular distribution of peroxidases may be studied by electrophoresis after extraction of the enzyme from cytoplasmic and wall-bound fractions.

Lipetz (1962) observed that lignification of sunflower crown gall tumors and other tissues from healthy stems and roots was influenced markedly by the concentration of specific ions in the medium. The isolation of crown gall peroxidases from cytoplasmic and cell wall fractions with calcium and other divalent cations was studied intensively by Lipetz and Garro (1965). They found that calcium, magnesium and ammonium ions were, in this order, most effective in affecting peroxidase release into the medium. Stafford and Bravinder-Bree (1972) studied tissue and intracellular localization of peroxidase isozymes of sorghum

by vacuum infiltration method and calcium extraction. They demonstrated that a significant proportion of the activity was associated with the cell walls, and that the major anodal isozymes were actually missing from the wall fraction. The vacuum infiltration method was also used by Birecka and Miller (1974). Contamination of cytoplasmic with wall-bound peroxidases will occur by this method even though it does not require the long time of incubation of the extraction medium with tissues. Stafford and Bravinder-Bree (1972) observed the activation of peroxidases by calcium ion when vacuum infiltration was used. It seems that this activation might not be caused by calcium ion *per se* but might be from the contamination. Calcium ion did not activate peroxidases according to Ranadive and Haard (1972). Maize peroxidases were also not activated by calcium ion as will be reported later. In the report of Stafford and Bravinder-Bree (1972), considerable amounts of cathodal peroxidases were associated with cytoplasmic fraction. They used very young sorghum internodes (3-4 day). In this stage of growth considerable levels of cathodal peroxidases still remain in cytoplasmic fraction. However, as plants become mature such peroxidase might become entirely wall-bound. This was confirmed in the experiment reported later.

5) Association of Peroxidases with Cell Particulates

In addition to wall-bound fraction, some peroxidase activity has been reported to be associated with mitochondria (Yonetani and Ohnishi 1967; Darimont and Baxter 1973), ribosomes (Lanzani and Galston 1964; Penon et al. 1970; Darimont and Baxter 1973) or chromosomes and nucleoli (Raa 1973). Penon et al. (1970) isolated three peroxidase isozymes R_1, R_1 and R_2 associated with the ribosomes of lentil roots by chromatography on CM-Sephadex C-50 and determined their spectral characteristics and

molecular weights. The de novo synthesis of two basic isozymes R_1 and R_1 was considerably stimulated by IAA treatment, while acidic isozyme R_2 was unaffected. Raa (1973) detected peroxidase activity in chromosomes, nucleoli, cell walls and cytoplasm by staining several plants with a mixture of p-phenylenediamine and hydrogen peroxide. He discussed the possible roles of peroxidases bound to different structures--i.e., he suggested that chromosomal peroxidases might have a histone-like function. However, before discussing any roles of these particule-bound peroxidases, it is necessary to affirm that they are bound enzymes rather than or contaminant. According to the report of Darimont and Baxter (1973), peroxidase isolated from ribosomes and mitochondria of lentil roots showed similar isozyme patterns, and much peroxidase activity dissociated from the ribosomes during sedimentation. This was suggested to be an indication that some isozymes were simply attached to the surface of ribosomal membranes and they were not ribosomal peroxidases. Plesnicar et al. (1967) demonstrated that the presence of peroxidases in mitochondria from etiolated mung bean hypocotyls and cabbage spadices resulted from contamination of the isolated mitochondria by a denser particle fraction with high peroxidase activity, and the mitochondria themselves had no peroxidase activity.

Regulation of Peroxidase Activity by Plant Growth Hormones

The effect of growth substances on peroxidase activity is of particular interest because peroxidase is known to catalyze the oxidation of IAA. Conversely, IAA alters the activity of peroxidase in plant tissues, suggesting that this mutual interaction may be important in the regulation of plant growth. For example, Ockerse et al. (1966)

reported that one of seven peroxidase isozymes detected from stem sections of dwarf peas developed as the tissue elongated and aged, and was correlated with a diminution in the supply of endogenous auxin.

Repression of peroxidase isozymes by IAA has been reported by Galston et al. (1968) in tobacco pith tissues, by Lavee and Galston (1968a) in pelargonium pith tissues and by Stuber and Levings (1969) in oat coleoptile. Whitmore (1971) reported that the formation of some peroxidase isozymes was inhibited by a low concentration of IAA (0.017 nM) in both soluble and wall fractions of wheat coleoptile tissues. Hydroxyproline at 1.0 nM prevented the IAA-induced inhibition. The mechanism in which hydroxyproline prevents the IAA-induced decrease in peroxidase and IAA oxidase activities is unknown. Hydroxyproline is reportedly a constituent of peroxidase (Shannon et al. 1966; Liu and Lamport 1968), but it is not known whether it is necessary for activity of the enzyme. Hydroxyproline is incorporated directly into protein when it is present at growth-inhibiting levels (Cleland and Olson 1967; Holleman 1967). Sirju and Wilson (1974) demonstrated that IAA between 10^{-4} to 10^{-2} M inhibited the increase in peroxidase and IAA oxidase activities with aging of potato. In a study of pelargonium pith tissues, the development of peroxidase activity was at first inhibited (up to ca. 50 hours in culture) and later promoted (ca. 100-150 hr. in culture) by IAA (Lavee and Galston 1968a). A similar biphasic effect of IAA upon pea peroxidase was reported by Sargent et al. (1974).

IAA is not the only plant hormone which affects the activity of peroxidase or IAA oxidase. The application of gibberellic acid (GA) to dwarf corn and pea produced no qualitative change in peroxidase isozyme

pattern, but increased the quantity of certain isozymes and decreased that of others (McCune 1961; Birecka and Galston 1970). Similarly, GA caused increases in three fast-moving IAA oxidases A_5, A_6 and A_7 in tobacco tissues, but the effectiveness of GA was dependent upon the concentrations of IAA and kinetin (Lee 1971b).

Yang (1967) discovered that an enzyme system of ethylene synthesis was strikingly similar to the peroxidative IAA oxidase system. Observations of interactions between auxins and ethylene have been made for many physiological processes (Burg 1962; Galston and Davies 1970; Steward and Krikorian 1971; Abeles 1972, 1973). The regulation of ethylene on the activity of peroxidases has, therefore, been thus studied intensively (Ku et al. 1967; Yang 1968; Mapson and Wardale 1968; Ku et al. 1970; Abeles 1973; Poovaiah and Rasmussen 1973; Sargent et al. 1974). Yang (1968) demonstrated the formation of ethylene from methional or a-keto-r-methylthiobutyric acid by HRP in the presence of Mn^{++}, SO_3^{--}, oxygen and a specific phenols. Fowler and Morgan (1972) reported the increase of peroxidase and IAA oxidase activities and internal level of ethylene after manganese treatment of cotton. The micronutrient manganese has been implicated as a cofactor in both the IAA-destroying system (Kenten and Mann 1953; Kenten 1955; Hillman and Galston 1956) and the ethylene-synthesizing system (Abeles and Rubinstein 1964; Yang 1967). The significance and mechanism of manganese in IAA oxidase system will be discussed later.

The action of IAA and other plant hormones on peroxidase activity are very diverse and variable. The effects depended on concentrations of applied hormones or plant sources and age (Lavee and Galston 1968b; Lee 1971c; Sirju and Wilson 1974). It seems that at least two possi-

bilities might be involved in those variability of the experimental results reported on hormonal action. First, all hormones applied affect on peroxidases indirectly. That is, they first produce ethylene, and the ethylene might be a repressor or derepressor of peroxidase synthesis. Quantitative increases of ethylene by applied IAA and its action are reported by Milborrow (1973), Abeles (1973) and Sargent et al. (1974). Secondly, all or at least some of hormone action is mediated by cyclic adenosine monophosphate (cAMP). Many data have accumulated concerning the involvement of cAMP on hormonal control in animal bodies (Happer 1971). Recently cAMP was also discovered in plant and its effect on plant hormones were reported (Kamisaka and Masuda 1970; Azhar and Murti 1971; Salomon and Mascarenhas 1971a, 1971b, 1972). However, even if plant hormonal action is mediated by cAMP the question as to the mode of action still remains.

IAA Oxidase Activity Peroxidases

Since Thimann (1934) reported the inactivation of native auxins by plant tissue homogenates, sufficient evidence has accumulated to define a peroxidase-based enzyme system known as IAA oxidase (Ray 1960; Shin and Nakamura 1962; Hare 1964; Meudt and Gaines 1967; Fowler and Morgan 1972). Ray (1960) showed that the IAA-oxidising enzyme preparation from Omphalia flavida exhibited peroxidase activity on pyrogallol with an optimum at pH of 3.5-3.7, similar to that for the IAA-oxidizing activity. The peroxidase and IAA-oxidizing activities were parallel both during thermal inactivation and in the course of enzyme purification. Therefore, it was assumed that only one enzyme was involved. Similarly, the peroxidase and IAA oxidase activities obtained from birch seedlings moved

together during membrane ultrafiltration, gel filtration and ion exchange chromatography, so it was concluded that both types of activity resided on one enzyme (Hoyle 1972). This view has been supported by the fact that purified HRP, in the presence of certain cofactors (Mn^{++} and phenols), catalyzes the oxidation of IAA without addition of hydrogen peroxide (Fox et al. 1965; Hinman and Lang 1965). Fox et al. (1965) added IAA to commercial HRP under aerobic conditions and observed the presence of intermediate compounds which were spectrally and kinetically similar to complex I and II formed from HRP and H_2O_2.

Evidence that peroxidase participates in the IAA-degrading system has been provided by using mutant plants. Several publications reported the increase of peroxidase activity in dwarf plants and slowly growing tissues Van Overbeek 1935; Galston and Dalberg 1954; Kamerbeek 1956; McCune and Galston 1959). Kamerbeek tested quantitatively the peroxidase content in various plants and found that swarf plants had a peroxidase contents higher than normal and that giant types gave exactly the reverse figure.

It is evident, then, that IAA oxidase activity is associated with the presence of a peroxidase. However, problems still remain concerning knowledge as to whether the enzymatically active site of the IAA oxidase is similar to that of the peroxidase. Related to this problem, there are some discrepant reports. All of the known plant peroxidases isolated in the pure or nearly pure form have been hemoprotein (Keilin and Mann 1937; Theorell 1940; Kondo and Morita 1952; Morita 1954; Morita and Kondo 1954; Yamazaki et al. 1956; Tagawa and Shin 1959; Tagawa et al. 1959; Shannon et al. 1966). Tang and Bonner (1947) reported that the IAA oxidase from etiolated pea epicotyls might be an iron protein or even a heme-containing

protein as a result of a carbon monoxide experiment. Three years later, however, Wagenknecht and Burria (1950) concluded that the IAA oxidase from roots of yellow wax beans and from etiolated pea epicotyls was a copper protein, not an iron protein. Their theory was based on the inhibition of the IAA oxidase by various copper chelating agents, especially sodium diethyldithiocarbamate (DIECA). In another study, Oaks and Shaw (1960) reported that peroxidase from Melampsora lini was mildly inhibited by DIECA, whereas the IAA oxidase was strongly inhibited. According to the report of Gortner and Kent (1953), however, IAA oxidase isolated from pineapple was not inhibited by copper enzyme inhibitors. Briggs et al. (1955) also found no influence of DIECA on IAA oxidase activity of extracts from Osmunda cinnamomea, and they stated that this IAA oxidase was an iron enzyme. These old reports gave rise to confusion concerning the ion nature of IAA oxidase protein. However, at least for two reasons the copper-protein theory of IAA oxidase seems to be the result of misinterpretation of data. First, the copper theory was based on the inhibition by DIECA. However, recent knowledge of science revealed that it did not chelate copper exclusively (Janssen 1970). Secondly, IAA is destroyed not only by peroxidase but other enzyme systems especially polyphenoloxidase (Briggs and Ray 1956; Konings 1964) and laccase (Fahraeus and Tullander 1956. Polyphenoloxidase and laccase are copper proteins (Barman 1969). When these enzymes are contaminated in the crude extracts more than peroxidase, they oxidize IAA more rapidly than peroxidase.

Older studies of peroxidase or IAA oxidase action usually assume that only one enzyme is responsible for the reaction. For many years the crystalline enzyme preparations have been shown to be of molecular

or isozymic heterogeneity (Klapper and Hackett 1956: Market and Moller 1959; Shannon 1968; Markert 1968; Scandalios 1969; Wilkinson 1970).

Endo (1968) and Yoneda and Endo (1969) analyzed the zymograms of commercial HRP and crude extracts of several plants regarding the dual catalitic activities of peroxidase and IAA oxidase isozymes. They found that all peroxidase isozymes were actually possessed IAA oxidase activity. Similar results were reported by Macnicol (1966), Kay et al. (1967) and Stafford and Bravinder-Bree (1972). Although the ratio of peroxidase and IAA oxidase activities of an isozyme or among isozymes varies, it seems that all peroxidase isozymes possess the IAA oxidase activity. This will be discussed in more detailed later.

Peroxidase and Lignification

The dehydrogenative enzyme system causing the polymerization of the p-hydroxycinnamyl alcohol to the polymeric lignin molecule forms one of the focal points in lignin biosynthesis. Of the three potential enzymes, polyphenoloxidases, laccases and peroxidases, the first group of enzymes was eliminated from consideration by studies of Mason and Cronyn (1955). These workers demonstrated that a purified mushroom polyphenoloxidase did not effect dehydrogenative polymerization of coniferyl alcohol.

Numerous studies on lignification in intact plants have demonstrated a direct relation between peroxidase level and lignification (Siegel 1953, 1954, 1956, 1957, 1962; Lyr 1957, Stafford 1965; Lipetz and Garro 1965; Ranadive and Haard 1972; Harkin and Obst 1973). Siegel (1953, 1954) demonstrated that the polymerization resulting from the action of Elodea densa tissues on eugenol was inhibited by IAA, which competitively

inhibited the oxidations catalyzed by peroxidase. He suggested that the high levels of auxin in rapidly elongating organs would supress peroxidase activity, and hence lignin deposition. With the decline of auxin concentrations that accompanies maturity, lignification would increase. Studies of lignin formation in vascular regions of coleus by Hepler et al. (1970) indicated that peroxidase was located in the secondary and primary cell wall regions where lignin deposition occurred.

It is more difficult to decide between laceases and peroxidases as polymerizing enzyme systems if we want to choose only one enzyme. In lignifying tissues, both enzymes can usually be shown to be present, although the peroxidase activity appeared to be more pronounced in bamboo shoots (Higuchi 1957). On the contrary, the activity of peroxidase in enzyme extracts isolated from spruce cambium was much weaker than that of laccase (Freudenberg et al. 1958). However, several factors induced many investigators to favor peroxidase as the lignifying mediators in plants. Those are 1) the lack of information on distribution and reaction mechanism of laccase in higher plants in contrast to well-documented data on peroxidase in many lignified species, 2) the ability of peroxidase and hydrogen peroxide to transform p-coumaryl alcohol into lignin-like polymers as well as laccase and air.

Haskins and Obst (1973) recently showed peroxidase to be apparently the only enzyme that polymerizes p-coumaryl alcohol to lignin in trees. They applied syringaldazine to various tree species. However, no purpling was evident on cross sections of tree branches or saplings or on cambial tissue culture unless hydrogen peroxide was added. They concluded that this indicated the absence of laccase but presence of peroxidase in lignifying cells. They used only one substrate in their

experiment. However, many compounds are known to be potential lignin precursors (Higuchi 1971; Sarkanen and Ludwig 1971). Involvement of other compounds will also become clear when the chemical structure of lignin is revealed. Laccase and even polyphenoloxidase might be involved in the oxidation of such compounds.

Effects of Ionizing Radiations on Peroxidases

Amounts of peroxidase or its isozyme patterns have been reported to be regulated not only during plant growth and differentiation Macko et al. 1967; Hamill and Brewbaker 1969; Gordon 1971; Lodha et al. 1974) but also during exogenous stresses. They are pathogenic infections (Lovrekovich et al. 1968; Seevers et al. 1971), wounding by cutting (Kawashima and Uritani 1965; Galston and Davies 1970), application of plant hormones (Rychter and Lewak 1971; Lee 1972) or ionizing radiations (Levitt 1972). The influence of different types of radiations on peroxidase activity has been the object of rather extensive studies. Similarly as in other stresses, peroxidase activity of irradiated plants is often higher than controls (Giacomelli 1967; Endo 167; Conklin and Smith 1969; Chourey et al. 1973). Changes of some peroxidase isozymes following irradiation have been reported (Haskins and Chapman 1956; Haskins and Downs 1961; Giacomelli and Cervigni 1964; Khudadatov et al. 1969; Fomenko 1971). Haskins and Chapman (1956) reported that corn seedlings which were dwarfed as a result of seed treatments with X-rays and thermal neutrons yielded enzyme preparations with higher activity than controls. These enzymes involved peroxidase, phosphatase, and polyphenoloxidase. Giacomelli and Cervigni (1964) studied the effect of different exposure rates (5.9 to 42.6 r/hr) of chronic gamma-

irradiation on peroxidase activity during the growth of Vicia sativa. Control plants showed peak peroxidase activity at the end of the flowering period, while in irradiated plants the maximum occurred several days earlier. This was particularly obvious in the plants irradiated at the highest exposure rate.

Endo (1967) reported a large increase of some proxidase isozymes and the appearance of a heavy trailing band (?) in anodal zone from internode drived from irradiated maize seeds. Whether the newly formed heavy trailing band was new "band" or just diffusate from other increased band is not clear. This will be discussed later. Appearance of several new peroxidases from tobacco hypocotyl and cotyledons after irradiation with X-rays or fast neutrons was reported by Conklin and Smith (1969).

Several explanations are possible for the activation of peroxidase or changes of their isozymes. However, it should be kept in mind that peroxidase was not the only activated enzyme but other enzymes were reportedly also changed (Haskins and Chapman 1956; Fomenko 1970). An increased activity of enzymes might be generally due to: 1) inactivation of natural inhibitors, 2) the release of activators, 3) an increase de novo synthesis of the enzyme, 4) the release of wall-bound or inactive enzymes. In all cases plant hormones may be actively involved.

Fomenko (1970) proposed that the inactivation of natural inhibitors was more important than de novo synthesis of peroxidases. Ogawa and Uritani (1970) suggested increased de novo synthesis of peroxidases through enhanced ethylene production. In contrast, Chourey et al. (1973) explained this by the removal of repression by IAA. It should be noted that the increase of peroxidase activity by radiations may not be caused by only one of mechanisms mentioned previously. Rather,

combinations of these mechanisms in addition to unknown (e.g., peroxide formation) may be involved. The reason is that none of the treatments mentioned was able to increase all peroxidases but did activate or form one or two isozymes. It is also apparent that there is no single mechanism to explain the change of peroxidases by radiations and other stresses. However, some of the mechanisms might be overlapping among them. This problem has been intensively reviewed by Levitt (1972).

GENERAL MATERIALS AND METHODS

Materials

Field corn (Zea mays L. var. H68 hybrid, or AA25 or AA8 inbred lines) was used as a source of seeds throughout the studies. Uniform and healthy seeds were selected for use.

Seedling root and mesocotyl tissues were obtained from dried seeds germinated and grown in the dark on moist paper. After a period of growth, the etiolated mesocotyls (first internode of maize stalks) and roots were harvested. The mesocotyls were divided into cortex and stele parts. The steles were stripped from the cortex by making a cut in the base of the cortex to expose the stelar thread, which could be pulled free according to the method of Stafford and Bravinder-Bree (1972).

Leaves and coleoptiles were obtained from seedlings grown in Jiffy-7 pots in the laboratory. Coleoptiles were usually obtained from the plants 5-7 days after sowing.

Mature leaves, pericarp and pollen grains were collected from plants grown at the Waimanalo Experimental Station and stored at -10°C until use.

Methods

1) Enzyme preparation

Plant tissues were ground in a chilled Sorval Omnimix blender with ten times their weight of 0.2 M phosphate buffer (pH 6.0). The homogenate was either strained through four layers of cheesecloth or filtered through Whatman filter paper No. 1. The filtrate was centrifuged at 12,000g for 10 min. The supernatant solution was collected and used as crude enzyme preparation. For further purification, the supernatant was brought to 35-75% ammonium sulfate fractionation. The resultant precipitate was

dissolved with 0.02 M phosphate buffer)pH 6.0), and dialyzed against the same buffer at 4°C. After centrifugation of the dialysate at 12,000g for 10 min, the supernatant was used as an enzyme source and stored at -10°C until use.

2) Isolation and purification of isozymes

After electrophoresis of the ammonium sulfate fractionated enzyme, a part of the gel was cut and stained for the estimation of each isozyme position. A gel containing an isozyme was cut out from the residual gel following an extraction of the isozyme by grinding the gel with 0.02 M phosphate buffer in a mortar. The mixture was then centrifuged at 12,000g for 10 min. The supernatant was concentrated with 90% ammonium sulfate and the precipitate produced was dissolved with 0.005 M Tris-HCl buffer (pH 8.4). After dialysis of the concentrate against the same buffer, it was transferred to a DEAE-cellulose chromatographic column previously equilibrated with the same buffer. The column was eluted with a linear gradient consisting of 500 ml of 0.005 M Tris-HCl buffer, pH 8.4, and 500 ml of 0.005 M Tris-HCl buffer (pH 8.4) containing 0.1 M NaCl according to the method of Shannon et al. (1966). Effluent fractions containing enzyme activity were pooled and used as an isolated isozyme source after concentration with AMICON B15.

3) Peroxidase assay

Peroxidase activity was measured by following colorimetrically (Baush & Lomb colorimeter) or spectrophotometrically (Unicam SP1800 Ultraviolet Spectrophotometer) the change in absorbancy (A) at 475 nm (guaiacol oxidation) or 460 nm (o-dianisidine oxidation) in the presence of hydrogen peroxide and enzyme. The guaiacol reaction mixture consisted of 5.4×10^{-4} M guaiacol, 3.5×10^{-5} M hydrogen peroxide, enzyme diluted

to give a linear reaction rate for at least 2 min, and 0.002 M phosphate buffer (pH 6.0) in 3.0 ml final volume. The complete system for the peroxidase assay by o-dianisidine contained 3.3 x 10^{-4} M o-dianisidine, 5.0 x 10^{-4} M hydrogen peroxide, the diluted enzyme and 0.02 M phosphate buffer (pH 6.0) in 3.0 ml final volume. In both systems, one unit of peroxidase activity was equivalent to the amount of enzyme which would cause an increase in absorbancy per min (A/min). The specific activity of the enzyme was expressed as unit per mg of protein, gram fresh weight or dry wall weight (A/min/mg protein, A/min/g fresh wt and A/min/g dry wall wt, respectively). o-Dianisidine was usually used as a substrate except in the study of pollen inhibitor where guaiacol was also used.

4) IAA oxidase activity

Indoleacetic acid oxidase (IAA oxidase) activity was determined by the colorimetric method. The reaction mixture consisted of 30 g IAA, 0.1 mM of 2 mM $MnCl_2$, 10 mM acetate buffer (pH 4.5), and enzyme solution. The mixture had a final volume of 2.0 ml, and was left to stand in the dark for 30 min. The reaction was stopped by adding 4 ml of Salkowski reagent (mixture of 50 ml of 35% $HClO_4$ and 1 ml of 0.5 M $FeCl_3$), and the remaining IAA content was determined by measuring absorbancy at 530 nm after 30 min.

5) Protein content

The Lowry method was used with crystalline bovine serum albumin as standard (Lowry 1951).

6) Electrophoresis of enzyme

Electrophoresis on 7% polyacrylamide gel and peroxidase staining with benzidine-H_2O_2 or o-dianisidine were conducted according to the method of Brewbaker et al. (1968). When eugenol was used for staining

gels, blue fluorescent bands were detected under short-wave ultraviolet light. The staining method for IAA oxidases is described in the results and discussion section.

7) Densitometric analysis of peroxidases

After staining the gel with o-dianisidine-H_2O_2, the gel was rinsed with 50% ethanol and then running water. It was then transferred to a Model 542 Densicord to trace the isozyme band using a 0.1 x 6 mm slit aperture and a 465 nm filter. The peak areas of each isozyme were estimated by triangulation.

8) Gamma-irradiation of seeds

Dried seeds were irradiated with a Cobalt-60 source (ca. 13,000 curies, April, 1974) at the Hawaii Research Irradiator in an air-filled central chamber at 25°C. The total absorbed doses varied from 0 to 500 krads. The dose rate for these experiments was 2.4 krads per min.

9) Paper chromatography of indole compounds

Paper chromatograms on Whatman No. 1 paper were developed in isopropanol-ammonia-water (10 : 1 : 1 by volume) according to the procedure of Sen and Leopold (1954). Indole spots on paper were detected by spraying 2% dimethylaminobenzaldehyde in 2 N HCl in 80% ethanol (Ehrlich's reagent) or 1% dimethylaminocinnamaldehyde in 6 N HCl and 95% ethanol (Bentley 1962).

RESULTS AND DISCUSSION

I. INTRACELLULAR LOCALIZATION OF PEROXIDASES

Peroxidases are highly polymorphic and ubiquitous in plant tissues, and their intimated functions are many and diverse. Maize genetic studies have revealed the genetic basis for isozyme variation of nine peroxidases among the twelve major peroxidases of this species. These twelve peroxidases are shown schematically in Figure 1, as they occur on 7% acrylamide gels at pH 8.1. Approximate Rf values are 74 for 9, 50 for 6 and 25 for 3. An additional enzyme, Px_E, has also been suggested in seed tissues (Brewbaker and Hasegawa 1974b).

These twelve peroxidases are highly tissue-specific. None of them has been detected in all tissues investigated (Table 1, Brewbaker and Hasegawa 1974a). This isozymic diversity suggests metabolic roles that differ slightly from one tissue to another. It is also probable that these enzymes are compartmentalized within cells, that is, one isozyme for the cytoplasmic fraction, one for the cell wall fraction and cellular particulates. Knowledge of the site of action of a specific isozyme may facilitate understanding of its physiological roles.

Extraction and Separation of Cytoplasmic and Cell Wall-bound Peroxidases

Extracts containing cytoplasmic or wall-bound peroxidases were obtained from various tissues of sweet corn (Zea mays L.) variety AA8. Each tissue was macerated for 3 min at high speed in a chilled Sorval Omnimix blender containing four times its weight of distilled water (Figure 2). The homogenate was filtered through Whatman filter paper No. 1. The residue remaining on the filter paper was later used for

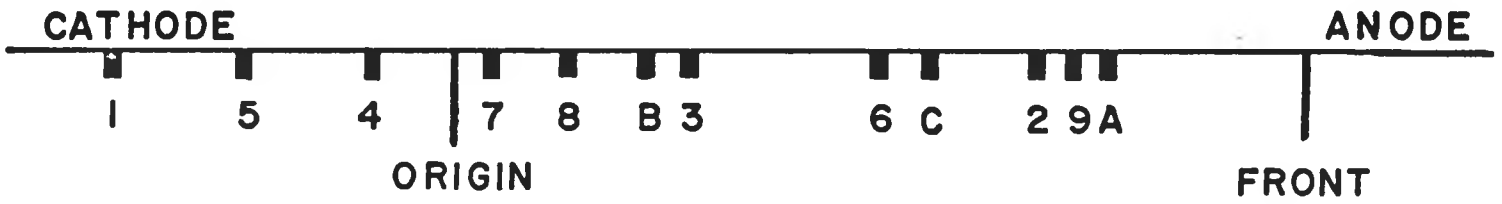


Figure 1. Approximate positions on 7% acrylamide gels at pH 8.1 of the major allelic isozymes of the 12 maize peroxidases.

Table 1. Tissue polymorphisms of the twelve maize peroxidases[1]

	RELATIVE ACTIVITY OF PEROXIDASE[2]											
TISSUE	1	2	3	4	5	6	7	8	9	A	B	C
LEAF	2	0	3	1	1	1	3	0	0	0	0	0
HUSK	1	0	3	1	1	2	3	0	1	0	0	0
GLUME (TASSEL)	1	0	2	1	0	1	3	0	0	0	0	0
COLEOPTILE	2	0	3	2	2	3	2	1	1	0	0	0
MESOCOTYL	2	0	3	2	1	3	2	2	1	0	0	0
CORTEX	1	0	3	2	1	2	1	1	0	0	0	0
STELE	1	0	0	0	1	0	0	3	1	0	0	0
PITH	1	0	0	2	1	2	0	3	0	0	0	0
STEM APEX	0	0	0	0	2	0	0	3	0	0	0	2
TASSEL INITIAL	0	0	0	0	1	0	0	3	0	0	0	3
EAR INITIAL	0	0	0	0	2	0	0	3	0	0	0	1
PERICARP (20 D)	1	0	2	2	2	0	1	1	3	0	0	2
EMBRYO "	0	0	0	1	0	0	0	2	1	0	0	3
ENDOSPERM "	1	0	0	0	3	0	0	0	1	0	0	2
ROOT	2	0	0	0	2	0	0	0	0	1	3	0
BRACE ROOT	1	0	2	0	1	1	3	0	0	0	3	0
CALLUS, STEM APEX	2	0	0	0	2	0	0	0	0	1	3	1
CALLUS, ENDOSPERM	1	0	0	0	0	0	0	0	0	1	3	1
SILK	1	0	0	3	0	0	3	0	0	0	0	0
ANTHER (2nd Mit)	0	0	0	0	3	0	0	0	0	0	0	2
POLLEN	0	3	0	0	1	0	0	0	0	0	0	3

1) Brewbaker and Hasegawa (1974a), modified.
2) Scale: 0 = no activity; 1 = low, 2 = medium, 3 = high activity.

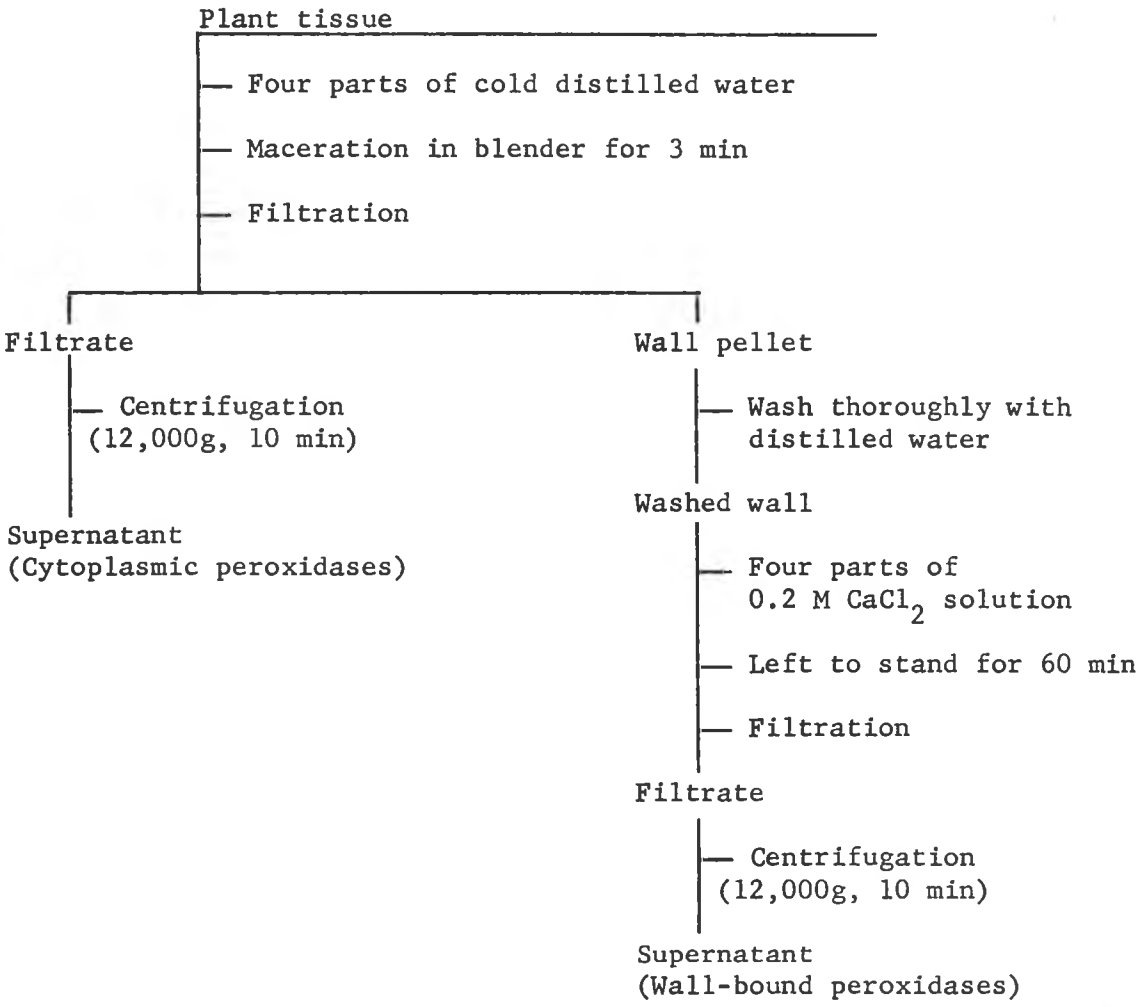


Figure 2. Flow chart of peroxidase extraction from cytoplasmic and wall-bound fractions of maize tissues.

extraction of wall-bound peroxidases. The filtrate was centrifuged at 12,000g for 10 min. The supernatant fraction was decanted and used as a source of "cytoplasmic peroxidases." Peroxidases were then extracted from isolated cell wall debris by thoroughly washing the residue on the filter paper until no peroxidase activity was detected in the washed solution. The washed cell wall debris was then collected and treated with three times original fresh weight of 0.2 M $CaCl_2$ for 60 min at room temperature. The mixture was filtered and centrifuged as above. The supernatant was decanted and used as the Ca^{++}-soluble or "exchangeable wall-bound peroxidases."

Exchangeable cell wall peroxidases are defined here as those removed by use of the calcium ion. Other ions such as K^+, Na^+ or Mg^{++} are known to be variously effective in this capacity (Lipetz and Garro 1965; Ranadive and Haard 1972). Lipetz and Garro (1965) reported the order of effectiveness to be $Ca^{++} \approx Sr^{++} > Ba^{++} > Mg^{++} > NH_4^+$. Calcium ion was much more effective in lower concentration, but K, Na or Mg ions were effective at higher concentrations (1 M) (Haard 1973; Moore 1973; Birecka and Miller 1974). Barium ion were avoided since all water- or acid-soluble barium compounds are poisonous to human beings (Stecker 1968). Magnesium ion has been reported to enhance peroxidase activity (Saunders et al. 1964). Sodium and potasium ions are well-known inhibitors of enzymatic oxidation of phenolic compounds (Barman 1969). Accordingly it seems advisable to use lower concentration of Ca ion rather than to use higher concentrations of other effective ions.

Experiments were performed to ensure that $CaCl_2$ facilitated the release of wall-bound peroxidases without activating the soluble enzyme.

The addition of equimolar $CaCl_2$ to the cytoplasmic fraction, or to the gel staining solution, had no measurable influence on total peroxidase nor on any individual isozymes. Ranadive and Haard (1972) also confirmed that the calcium ion had no effect on peroxidase activity when added during the enzyme assay.

Effect of Calcium ion Concentration and Extraction Time on Peroxidase Release from Cell Wall Fraction

The capacity of calcium ion to release peroxidases from wall fraction was shown to be dependent on its concentration and incubation time. Figure 3 shows that an increase of the concentration of $CaCl_2$ incubated with isolated cell walls facilitated greater release of peroxidases in the resulting supernatant fraction. The enzyme activity increased with $CaCl_2$ to a concentration of 0.2 M. The data suggest but do not prove a downward trend above 0.2 M. Ranadive and Haard (1972) reported similar results in their study of pear fruit peroxidase.

The rate of peroxidase release from wall fractions was shown to be dependent on incubation time with calcium ion (Figure 4). Peroxidase activity released from wall fractions increased progressively with incubation time up to 60 min.

A peroxidase zymogram of maize coleoptile of 7 day-old seedlings from cytoplasmic and wall-bound fractions is shown in Figure 5. Among eight peroxidase isozymes detected clearly, anodal enzymes 3, 6, 7, 8 and 9 were mainly cytoplasmic, while cathodal enzymes 1, 4 and 5 were largely in the wall-bound fraction.

Ca^{++}-soluble versus Ca^{++}-insoluble Peroxidases in Walls

Peroxidases in wall fractions have been reported to be bound to the

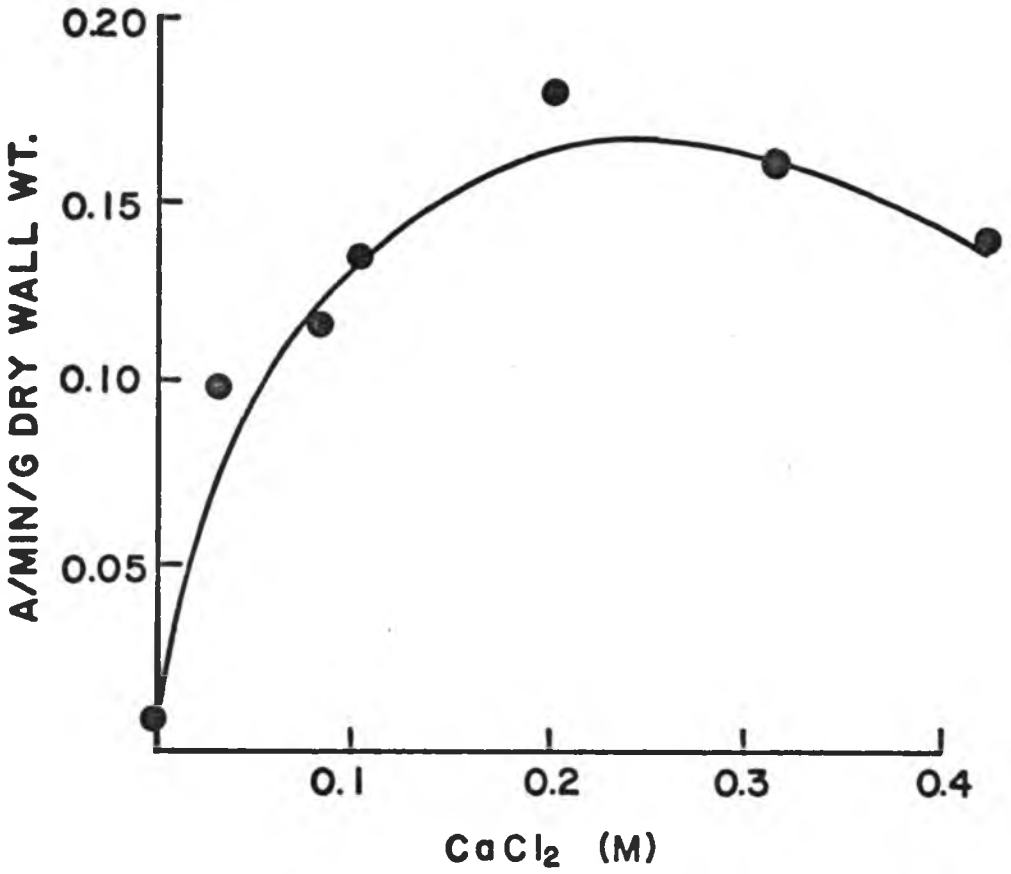


Figure 3. Effect of calcium ion on peroxidase release from isolated cell wall of 15 day-old maize leaves.

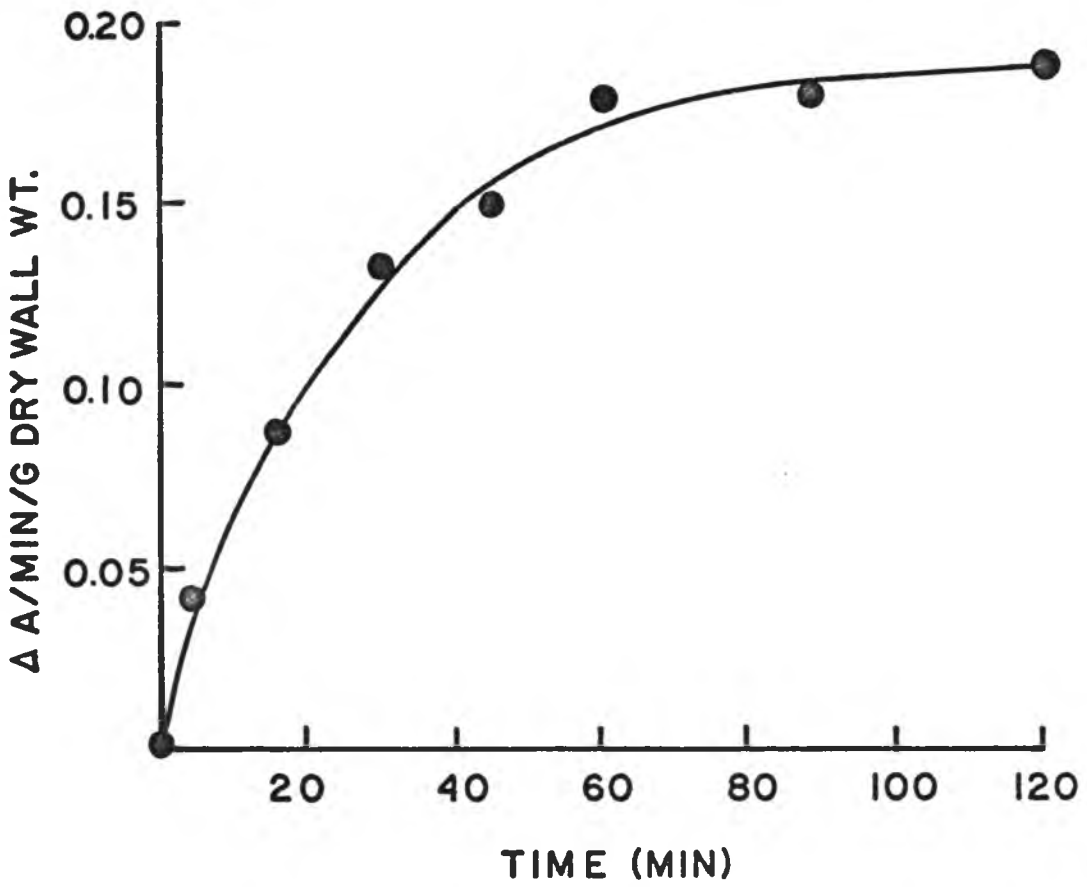


Figure 4. Effect of time of incubation with isolated cell walls of 15 day-old leaves and 0.2 M $CaCl_2$ on peroxidase release.

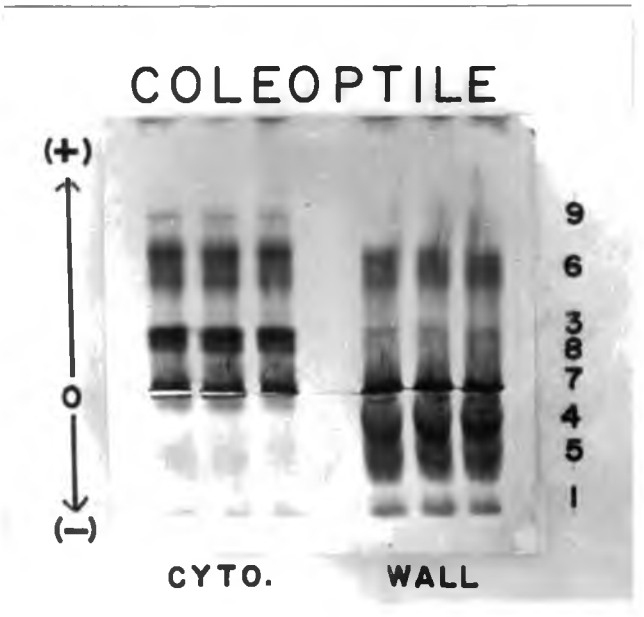


Figure 5. Zymogram of peroxidase isozymes in cytoplasmic (CYTO) and wall-bound (WALL) fractions of 7 day-old coleoptiles.

wall both ionically and covalently (Ridge and Osborne 1970; Hayward et al. 1973; Birecka and Miller 1974). Ionically-bound peroxidases are released by treating walls with some cations. The amounts of Ca^{++}-soluble (ionically-bound) and Ca^{++}-insoluble (covalently-bound) peroxidases were calculated for isolated cell walls of 15 day-old leaves, mesocotyls and roots (Table 2). After release of Ca^{++}-soluble wall peroxidases by treatments with 0.2 M $CaCl_2$ for 60 min, the residue was thoroughly washed with cold distilled water. An estimation of Ca^{++}-insoluble peroxidases was made by suspending the dried residue into the assay mixture and analyzing the solubilized product colorimetrically (following Stafford and Bravinder-Bree (1972) and Hayward et al. (1973)). In these tissues 90-94% of wall peroxidases was ionically-bound (Table 2). Roots appeared to contain higher activity of Ca^{++}-insoluble peroxidases than leaves or mesocotyls. Another method of Ca^{++}-insoluble enzyme release is to treat wall residues with cellulase-pectinase mixtures (Ridge and Osborne 1970; Birecka and Miller 1974), although results of this technique can be equivocal (Denna 1974). Covalently-bound peroxidases are commonly found in very low quantities compared to ionically-bound peroxidases. As with corn seedlings, the level of ionically-bound peroxidases was about 90% in lily pollen wall (Hayward et al. 1973), and was 70% in wall of soybean callus tissue (Moore 1973), 75% in wall of pea epicotyl (Rdige and Osborne 1970), and 96% in sorghum first internode wall (Stafford and Brevinder-Bree 1972). These values varied depending on plant age, tissue, or experimental conditions such as hormonal treatments (Birecka and Miller 1974).

Table 2. Amounts of Ca^{++}-soluble and Ca^{++}-insoluble peroxidases in walls of 15 day-old maize tissues

Tissues	Wall peroxidase activity in A/min/g dry wall wt	
	Ca^{++}-soluble	Ca^{++}-insoluble
Leaves	6.53 (93.0%)	0.49 (7.0%)
Mesocotyls	6.66 (93.9%)	0.43 (6.1%)
Roots	9.01 (90.3%)	0.97 (9.7%)

Physiological Significance of Peroxidases in Cytoplasmic and Wall-bound Fractions

Peroxidases were extracted from cytoplasmic and cell wall fractions and electrophoresed. Gels were incubated in o-dianisidine and hydrogen peroxide at pH 6.0 for one hour, and then rinsed with 50% ethanol. As an example, the cytoplasmic and wall-bound peroxidase isozymes from root extracts (Figure 6) produced densitometric tracings of Figure 7. After measuring protein content of extracts and estimating peak areas of each enzymes, the activity of each enzyme was calculated.

Intracellular distribution of twelve peroxidase isozymes from various maize tissues was quantitized from the zymogram tracings and the results are summarized in Table 3. Activity of total peroxidases or of individual isozymes varied greatly from tissue to tissue (Table 3). The highest total peroxidase activity was detected in roots among six tissues studied. This was also observed in ontogenetic and irradiation studies of these tissues, as discussed later. Leaves, coleoptiles and mesocotyls showed very similar values for total peroxidase activity (Table 3). However, there were large differences in their isozymes patterns and amounts, suggesting different metabolic roles of each isozyme among various tissues.

Nearly 50% of total peroxidase activity in leaves and mesocotyls was contributed by enzyme 3 which contributed only 20% of the activity in coleoptiles (Table 3). Isozyme 6 was much more active in the coleoptiles than in any other tissues. It contributed about 30% of the total peroxidase activity in the coleoptiles, while such a contribution of the enzyme 6 in the leaves and mesocotyls was 6% and 3%, respectively (Table 3). The coleoptile is the first leaf in monocots which sheaths

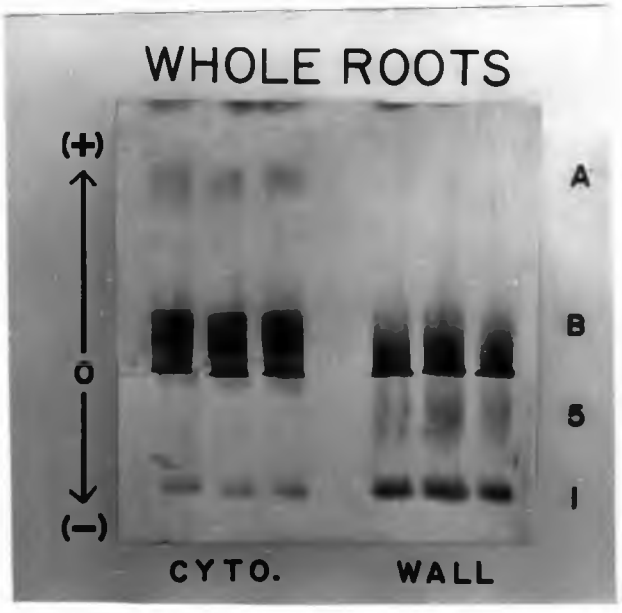


Figure 6. Zymogram of peroxidase isozymes in cytoplasmic (CYTO) and wall-bound fractions of 15 day-old whole roots.

Figure 7. Densitometric tracings of peroxidase isozymes in cytoplasmic and wall-bound fractions of whole roots.

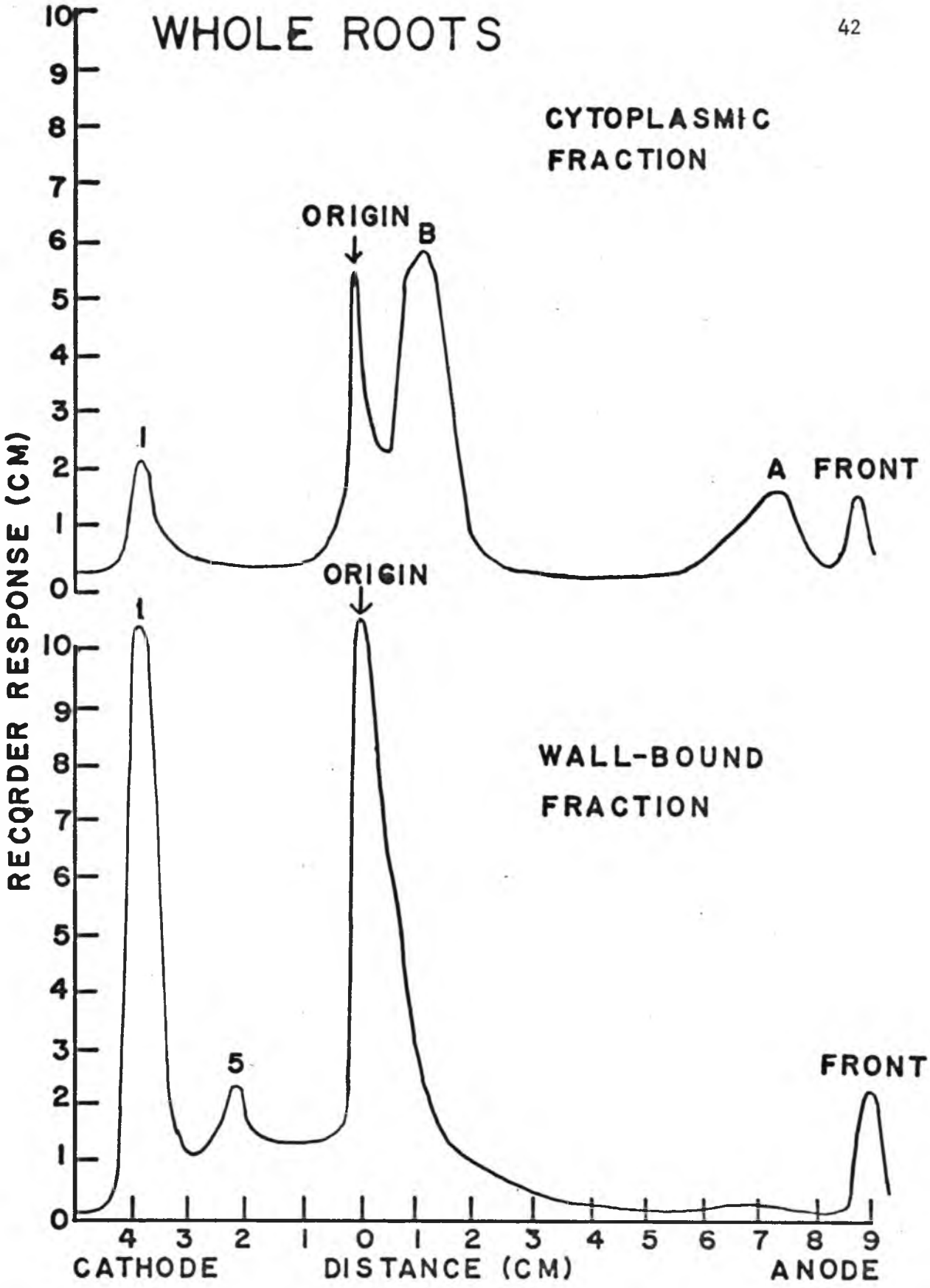

WHOLE ROOTS
CYTOPLASMIC FRACTION
ORIGIN
B
1
A
FRONT
ORIGIN
1
WALL-BOUND FRACTION
5
FRONT
RECORDER RESPONSE (CM)
0 1 2 3 4 5 6 7 8 9 10
4 3 2 1 0 1 2 3 4 5 6 7 8 9
CATHODE
DISTANCE (CM)
ANODE

Table 3. Amounts of twelve peroxidase enzymes in cytoplasmic (C) and wall (W) fractions of maize tissues

Tissues			Peroxidase activity in ΔA/min/g fresh weight											
			1	2	3	4	5	6	7	8	9	A	B	C
Leaf**	C	16.47 (70.4)	0.13 (0.6)	---	11.45 (48.6)	trace*	---	1.47 (6.3)	3.56 (15.2)	---	---	---	---	---
	W	6.92 (29.6)	3.05 (13.1)		0.20 (0.9)	0.25 (1.1)		trace	3.38 (14.5)					
Coleoptile**	C	14.58 (70.0)	0.41 (2.0)	---	4.64 (22.3)	0.00	0.44 (2.1)	5.07 (24.4)	3.13 (15.0)	---	0.91 (4.4)	---	---	---
	W	6.25 (30.0)	0.29 (1.0)		0.35 (1.7)	2.46 (11.8)	1.57 (7.6)	0.74 (3.6)	0.77 (3.7)		0.09 (0.4)			
Mesocotyl**														
Cortex	C	10.61 (71.0)	0.10 (0.7)	---	10.04 (67.2)	0.00	---	0.25 (1.7)	---	---	0.21 (1.4)	---	---	---
	W	4.33 (29.0)	1.82 (12.2)		0.57 (3.6)	1.87 (12.5)		0.08 (0.6)			0.00			
Stele	C	6.42 (71.2)	0.58 (6.4)	---	3.94 (43.6)	---	0.00	0.39 (4.3)	---	1.35 (14.9)	0.18 (2.0)	---	---	---
	W	2.62 (28.8)	0.38 (4.2)				0.26 (0.5)	trace		1.93 (21.4)	0.00			
Root**	C	24.84 (72.9)	3.32 (9.8)	---	---	---	0.00	---	---	---	---	5.64 (6.6)	15.89 (46.6)	---
	W	9.24 (27.1)	6.58 (19.3)				2.66 (6.6)					0.00	0.00	
Pericarp***	C	9.23 (75.4)	0.00	---	2.44 (19.9)	0.00	0.00	---	0.42 (3.4)	0.08 (0.7)	6.20 (49.0)	---	---	trace
	W	3.02 (24.6)	0.13 (1.1)		0.36 (3.0)	1.02 (8.3)	0.53 (4.3)		0.56 (4.6)	0.29 (2.4)	trace			0.10 (0.8)
Pollen***	C	31.20 (99.0)	---	24.83 (78.8)	---	---	1.99 (6.3)	---	---	---	---	---	---	4.11 (14.0)
	W	0.32 (1.0)		0.00			0.26 (0.8)							0.06 (0.2)

*) Specific activity below 0.05, **) From 15 day-old seedlings, ***) From field

succeeding leaves and first tissue to be exposed to sunlight. The uniqueness of coleoptiles and relatively higher activity of their enzyme 6 might be related to a plant growth regulation system involving the light-sensitive phytochrome system. Enzymes 3 and 6 almost always appeared together in lefy tissues (Brewbaker and Hasegawa 1974, also see Table 1). Although amounts of these two enzymes varied among tissues, the total amounts were not greatly different; e.g., 15.31 in mesocotyls, 13.12 in leaves and 10.80 in coleoptiles (Table 3). This may imply some relationship between enzymes 3 and 6. The ratio of these two enzymes could be regulated according to physiological requirement of each tissue. Selective regulation of peroxidase isozymes by plant hormones has been reported by several workers (Galston et al. 1968; Stuber and Levings 1969; Lee 1971a, 1971b, 1971c; Whitmore 1971; Lee 1972). Enzyme 3-null mutants are not yet known but a null mutant for enzyme 6 is known (Brewbaker and Hasegawa 1974). In such mutants enzyme 3 might assume additional roles otherwise held by enzyme 6. The extremely broad substrate-specificity of enzyme 3 (Table 5) implies this.

The activity of cytoplasmic and wall-bound fraction of the twelve peroxidases of maize are summarized in Table 3. Cathodal peroxidases 1, 4 and 5 were found largely in the wall-bound fraction. Especially enzymes 4 and 5 were exclusively localized in the wall fraction among tissues studied except pollen grains (Table 3). Enzyme 1 was also mainly found in the wall-bound fraction, although some amounts of it were detected in cytoplasmic fraction.

Anodal peroxidases were generally localized in the cytoplasmic fraction (Table 3). Among them enzymes having Rf values of more than

25 (enzymes 3, 6, 9, A. B and C) were almost exclusively detected from the cytoplasmic fraction. However, enzyme 8 and the non-migrating enzyme 7 about equally distributed between the two fractions (Table 3).

Each peroxidase isozyme could assume different physiological roles in relation to its distribution in different tissues or cell compartments. Well-bound peroxidases like maize enzymes 1, 4 and 5 could be involved in the synthesis of wall components, and especially of lignin. These enzymes were largely wall-bound in normal plants but were observed to be in very low amounts in leaves of low lignin mutants (Table 4). The mutant plants used were brown midrib-3 (bm_3) and brittle stalk-2 (bk_2). Enzymes 1, 4 and 5 were found in considerably higher amounts in the wall fraction in normal tissues such as leaves, coleoptiles or mesocotyls (Table 3). In contrast, these enzymes in the wall of the mutants were almost in trace amounts except enzyme 1 in bk_2 mutant. Wall enzymes accounted for about 30% of the total peroxidase activity in the normal plants, and for only 16% those in mutants plants (Table 4).

Genetic and chemical studies of the brown midrib mutants have been conducted by several workers (Kuc and Nelson 1964; Gee et al. 1968; Muller et al. 1971; Lechtenberg et al. 1972). Biochemical studies of brittle stalk have not yet been carried out. Leaves and stalk of bk_2 are very brittle and easily broken (Neuffer et al. 1968), suggesting lower amounts of wall components like lignin. Table 4 suggests the involvement of wall-bound peroxidases in lignification and other wall component synthesis. Involvement of peroxidase enzyme in lignification has been suggested by many studies, but critical studies were lacking (Siegel 1953, 1954; Kuc and Nelson 1964; Harkin and Obst 1973). According to Kuc

Table 4. Amounts of cytoplasmic (C) and wall-bound (W) peroxidases in leaves of brown midrib-3 (bm_3) and brittle stalk-2 (bk_2)

		Peroxidases activity in A/min/g fresh wt					
		Total (%)	1	3	4	6	7
Mutant plants	C	43.29 (84.2)	1.29	18.71	0.22	0.27	22.81
bm_3 leaf	W	8.34 (15.8)	trace*	trace	trace	trace	8.34
	C	53.45 (84.5)	2.93	24.63	0.24	0.60	25.05
bk_2 leaf	W	9.80 (15.5)	3.94	trace	trace	trace	5.86
Normal plants**	C	16.47 (70.4)	0.13	11.45	trace	1.47	3.56
AA8 leaf	W	6.92 (29.6)	3.05	0.20	0.25	trace	3.38
	C	14.58 (70.0)	0.41	4.64	0.00	5.07	3.13
AA8 coleoptile	W	6.25 (30.0)	0.29	0.35	2.46	0.74	0.77

*) Specific activity of enzyme below 0.05

**) see Table 3

and Nelson (1964), the brown midrib-1 mutant had considerably less p-hydroxycinnamic acid, one of the important lignin precursors. Although they did not analyze peroxidase activity, their data suggest the relationship of peroxidase activity to low levels of substrate. This mutant may be suitable material for the research on substrate-inducibility of peroxidases. Raa (1973) proposed that cell wall peroxidases degraded exogenous IAA, which might interfere with IAA-controlling processes within a cell. However, peroxidase requires a specific monophenol and manganese ion to destroy IAA (Hare 1964). Manganese usually serves as an activator of many enzymes in the cytoplasmic fractions (Barman 1969). It is not likely that a sufficient amount of these free cofactors of IAA oxidase is available in the wall fraction.

Cytoplasmic peroxidases might be actively involved in several cell processes such as regulation of growth by limiting the amount of IAA present, photosynthetic system, respiration process or energy transfer as discussed later.

From the results presented here, twelve maize peroxidases are classified into two groups. One group is anodal and mainly cytoplasmic, while another group is cathodal and largely wall-bound. In addition to these differences, the two groups also differed in several characters such as ontogenetic behavior, pH optimum values and heat sensitivity as will be discussed later.

II. ONTOGENETIC VARIATIONS OF PEROXIDASE ISOZYMES

Extensive empirical observations have been made of peroxidases during maize development, and essentially all tissues show a general pattern of increasing number and intensity of isozymes (McCune and Galston 1959; Hamill and Brewbaker 1969; Scandalios 1969; Lodha et al. 1974). Ontogenetic variations of peroxidases may be closely related to metabolic events at different stages of development.

Studies were made of developmental variations in the total activity and isozymes of maize peroxidases. Total soluble peroxidase activity increased significantly in seedling leaves, mesocotyls and roots between 5 and 15 days after sowing (Figure 8). Although peroxidase activity was observed to increase with development in leaves, mesocotyls and roots, the rate of the increase differed greatly among tissues. Increase of the peroxidase activity in roots was most rapid, followed by that of mesocotyls and leaves (Figure 8). Cotoplasmic and wall peroxidases in roots showed the highest activity at 15 days compared to other tissues (Table 3). Mesocotyls showed higher peroxidase activity than leaves during development (Figure 8). However, Table 3 shows very similar values for peroxidase activity at 15 days between mesocotyls and leaves. Activity was expressed as unit per g fresh weight in Table 3 and as unit per mg protein in Figure 8. The amounts of peroxidases in mesocotyls and leaves did not appear to be different.

The ontogenetic changes of cytoplasmic (soluble) peroxidase isozymes in the developing mesocotyl are presented zymographically in Figure 9, and densitometric tracings presented in Figure 10. From these Figures it was apparent that enzymes 3 and 7 increased very rapidly with

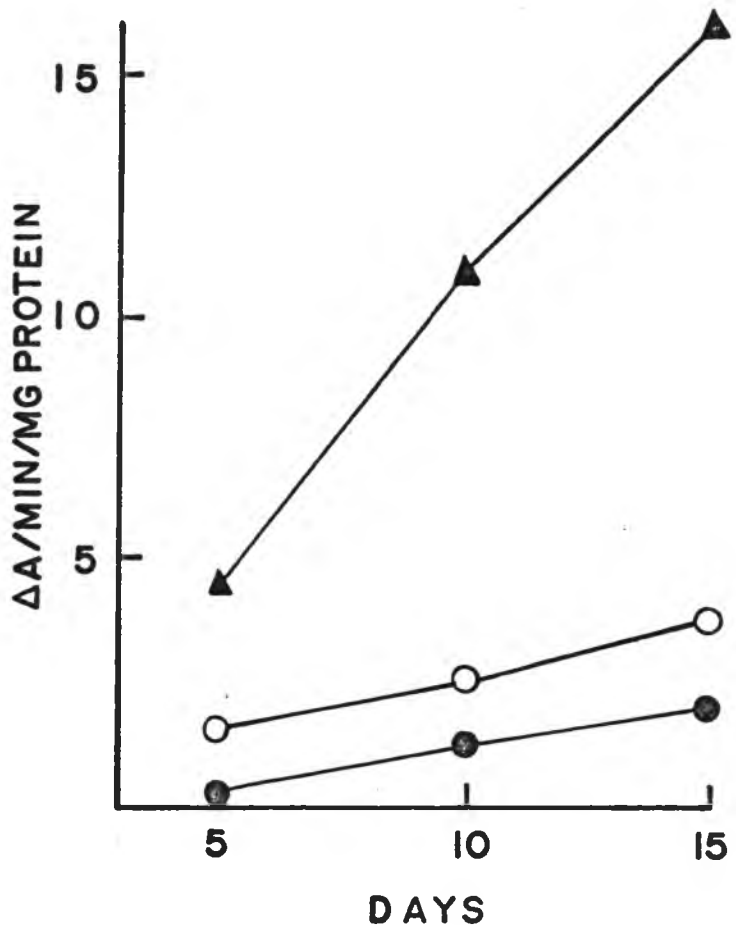


Figure 8. Changes of soluble peroxidase activity during growth of roots (▲—▲), mesocotyls (○—○) and leaves (●—●).

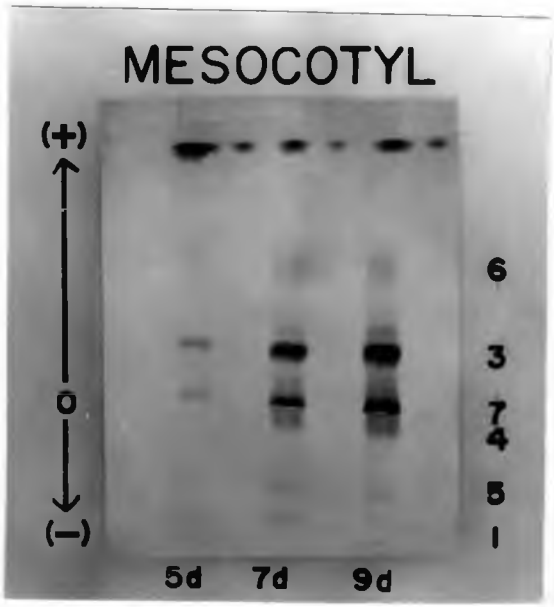
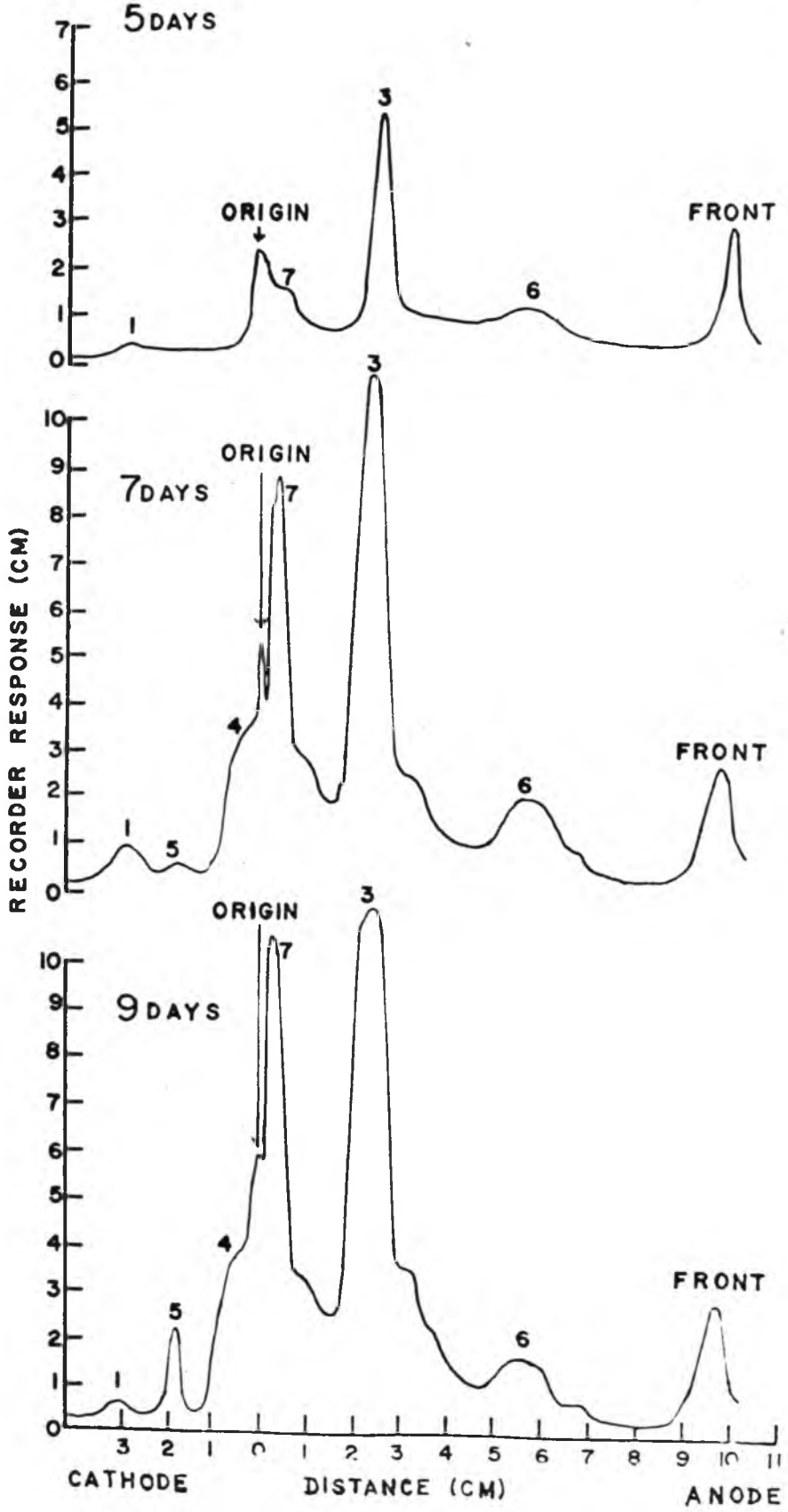


Figure 9. Zymogram of cytoplasmic peroxidase isozymes in the developing mesocotyls from 5 to 9 day-old seedlings.

Figure 10. Densitometric tracing of peroxidase isozymes in Figure 9.


5DAYS
ORIGIN
FRONT
7DAYS
ORIGIN
FRONT
9DAYS
ORIGIN
FRONT
RECORDER RESPONSE (CM)
CATHODE
DISTANCE (CM)
ANODE

age. The increase of total soluble peroxidase activity in developing mesocotyls (Figure 8) may be mainly related to the progressive and large increase of these enzymes. In addition to these two enzymes, enzyme 6 also increased but to less extents during development of the mesocotyls (Figures 9 and 10). Enzyme 1 appeared between 5 and 9 days but it gradually disappeared thereafter from the cytoplasmic fraction. Enzymes 4 and 5 were not observed at 5 days, but they were detected in considerably amount between 7 and 9 days (Figures 9 and 10). These enzyme, however, could not be detected from the cytoplasmic fraction thereafter.

The ontogenetic changes of both cytoplasmic and wall peroxidases in mesocotyls are presented zymographically in Figure 11. Cathodal enzymes 1, 4 and 5 were detected in the cytoplasmic fraction in early stage of development, but gradually disappeared from the cytoplasmic fraction. These enzymes in the wall fraction increased with age (Figure 11). The effect of age was very pronounced in the increase of enzyme 1 in the wall fraction. Enzymes 4 and 5 also increased gradually in the wall fraction. Figures 9, 10 and 11 indicate that cathodal enzymes 1, 4 and 5 were both cytoplasmic and wall-bound in the early stages of development. Some histochemical studies have demonstrated that peroxidase localization was cytoplasmic in the younger stage and associated with cell walls in the mature cells (De Jong 1967; Raa 1973).

Several explanations are possible why enzymes 1, 4 and 5 disappeared from cytoplasm and became wall-bound with age. Wall-bound peroxidases may be positively-charged proteins, since they were released only by cation treatments (Lipetz and Garro 1965; Ranadive and Haard 1972). They bind with negatively-charged wall components. Amounts of such wall

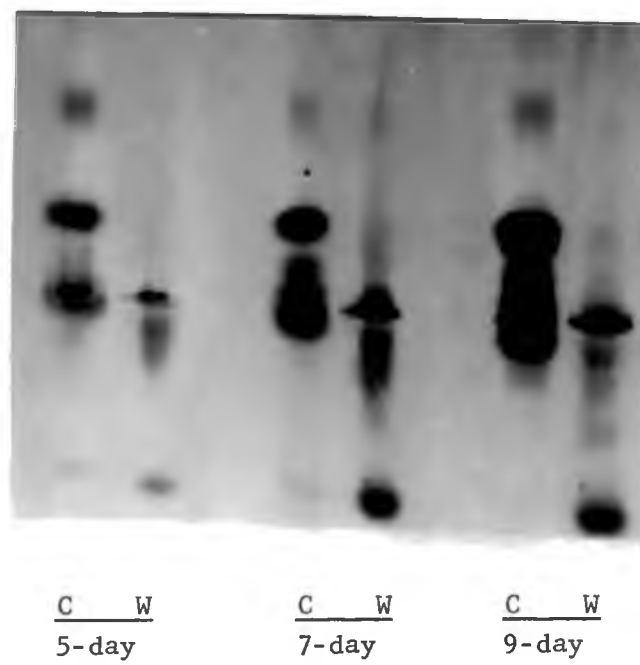


Figure 11. Zymogram of cytoplasmic (C) and wall (W) peroxidase isozymes in the developing mesocotyls from 5 to 9 day-old seedlings.

components like pectic substances are very low in developing younger cells (Leopold 1964) and only part of isozymes synthesized will be bound. Auxin is reportedly associated with cell wall synthesis (Davis 1973). This plant hormone could be actively involved in the regulation of wall peroxidase synthesis.

III. THERMAL SENSITIVITY, SUBSTRATE SPECIFICITY AND pH RESPONSE OF PEROXIDASE ISOZYMES

Thermal Sensitivity of Peroxidase Isozymes

Studies of thermal inactivation of maize peroxidases and of their regeneration in time were conducted with seedling tissues. The crude cytoplasmic extracts were prepared from leaves and coleoptiles of 7 day-old seedlings with ten times their weight of 0.2 M phosphate buffer (pH 6.0). The extracts were then heated in a water bath at $77^{o}C \pm 0.5$ for 30 seconds. Under these conditions it was found that all isozymes detected in the control remained active after heating.

Total soluble peroxidase activity in the extracts was measured at 20 min intervals after heating, and the percentage of regeneration was calculated (Figure 12). The regeneration of the peroxidase activity was very rapid within first two hours but was gradual, thereafter. The regeneration did not regeneration did not reach 100% after 24 hours of incubation at room temperature (Figure 12).

The changes of individual isozymes at each reading is presented zymographically in Figure 13. Eight peroxidase isozymes were clearly detected on the zymogram. Immediately after heating, activities of cathodal enzymes 1, 4 and 5 were detected, 1 and 5 in trace amounts only. Enzyme 5 did not regenerate after 24 hours of incubation. Gradual recovery of enzyme 4 and, to a less extent, enzyme 1 were observed (Figure 13). Among anodal peroxidases, enzyme 9 showed the least heat stability and no appreciable regeneration during incubation. Other anodal enzymes 6, 7 and 8 were observed to regenerate gradually but not completely under the experimental conditions. Enzyme 3 was the most

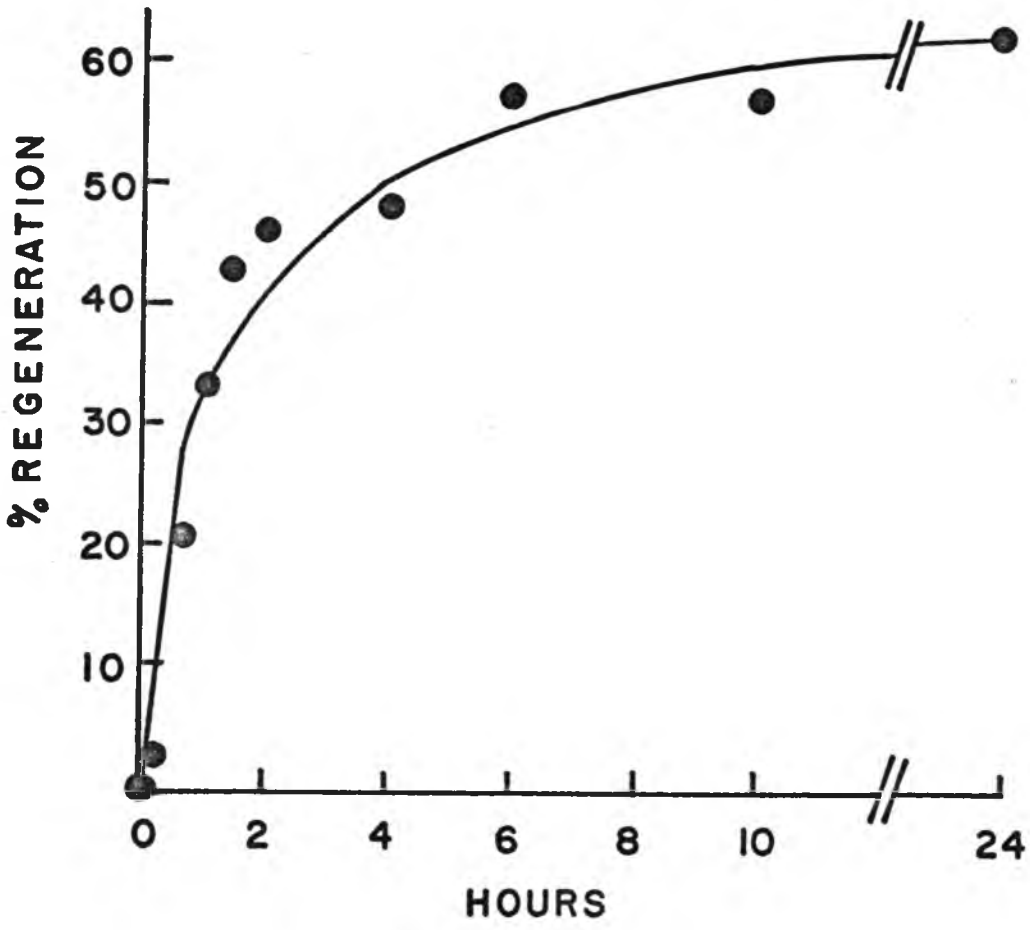
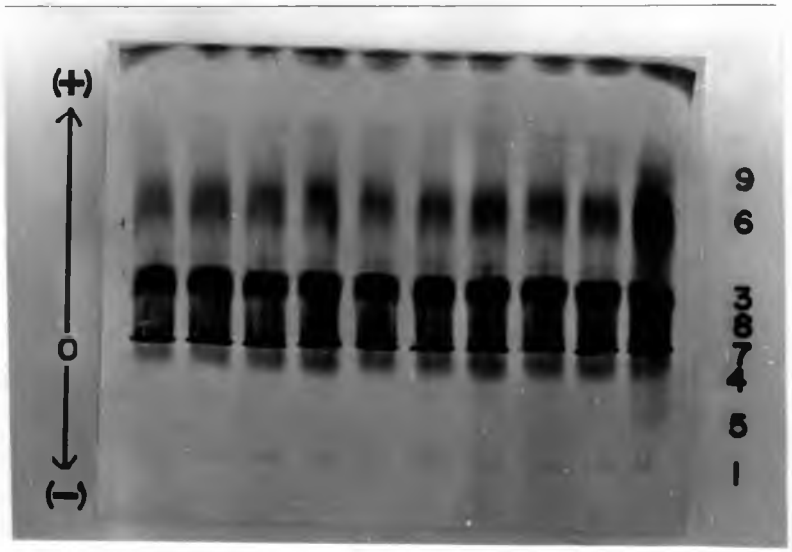


Figure 12. Regeneration of thermally inactivated peroxidase in mixtures of maize leaves and coleoptiles of 7 day-old seedlings.



0, 20, 40, 60, 90, 120, 240, 360, 600, C

Time after heating
(min)

Figure 13. Regeneration of thermally inactivated peroxidase isozymes of maize leaves and coleoptiles from 7 day-old seedlings.
(C = Non-heated control)

heat stable. Visually there was no difference in amounts of enzyme 3 before and immediately after heating (Figure 13).

The observed heat sensitivity differences among the enzymes does not appear to be related to the amounts of each enzyme present in the original extracts. Enzyme 9 existed at its greatest concentration in the corn pericarp, but was most rapidly inactivated by comparison to other enzymes of lower concentrations (Chenchin and Yamamoto 1973, enzyme 9 was numbered as A-5 in their study). Anodal enzymes are relatively heat stable except enzyme 9. All cathodal enzymes were heat labile with an exception of enzyme 4 (Figure 13). Heat-labile enzyme 9 is present in a rather small proportion of corn inbreds studied, and a null allele at this locus is much more frequent (Brewbaker and Hasegawa 1974b). There is no critical research reported concerning the heat lability of wall-bound peroxidases, but this has been substantiated by general studies (Gordon and Alldridge 1971).

Substrate Specificity of Peroxidases

Substrate specificity tests were conducted to provide further evidence relating to the different properties of the twelve maize peroxidases. Peroxidase was extracted from five tissues--leaves, coleoptiles, mesocotyls, roots and pollen. It was then resolved into isozymes on gel with different phenolic compounds and hydrogen peroxide. Phenolic compounds used as substrates were benzidine, o-dianisidine, eugenol, pyrogallol, catechol and guaiacol. After electrophoresis, the gels were incubated in the appropriate phenol solutions (0.01 M) in phosphate buffer at pH 6.0 and room temperature for 30 min. After observing the bands of polyphenoloxidases (o-diphenol oxidases) and

laccases (p-diphenol oxidases), hydrogen peroxide was added. The development of peroxidase bands was compared and the intensity of the reaction estimated visually 30 min after the peroxide was added (Table 5). In the assay of substrate specificity, the standardization of staining time was very important. The kinetics of color development and subsequent fadings, especially in benzidine, were highly variable with different substrates.

Benzidine, o-dianisidine, guaiacol and pyrogallol are often used for assays of plant peroxidases. Benzidine and o-dianisidine were good substrates for staining all maize peroxidase isozymes. They produced identical results for the twelve peroxidases (Table 5). In contrast, pyrogallol and guaiacol were not suitable substrates for visualization of all maize peroxidases, and staining was slow on each (Table 5). These two substrates have often been reported to be very poor substrates for peroxidase isozymes on gels (Farkas and Stahmann 1966; Rychter and Lewak 1971; Seevers et al. 1971), however, when isolated isozymes are used, they oxidized both substrates fairly well (McCune 1961; Kawashima and Uritani 1965). Several explanations are possible for these discrepancies. Higher concentrations of hydrogen peroxide are used for gel staining, and can specifically inhibit guaiacol or pyrogallol oxidation by some isozymes. Removal of cofactors during electrophoresis might make some isozymes inactive to oxidation. Benzidine, o-dianisidine and guaiacol are not naturally-occurring but artificial dyes (Harborne 1964).

Among the natural phenolies used as substrates, eugenol (a presumed lignin precursor) provided most definitive results. Wall-bound enzymes

Table 5. Substrate specificity of twelve peroxidases in maize

Substrate (0.01 M)	Relative activity of peroxidases as benzidine 100%											
	1	5	4	7	8	B	3	C	6	2	9	A
	In the presence of hydrogen peroxide (0.02 M)											
Benzidine	100	100	100	100	100	100	100	100	100	100	100	100
o-Dianisidine	100	100	100	100	100	100	100	100	100	100	100	100
Guaiacol	100	30	67	100	25	20	50	0	0	75	0	0
Pyrogallol	100	100	0	75	0	0	50	0	0	100	0	0
Eugenol	200	133	100	125	75	60	75	0	0	125	0	0
Catechol	50	33	33	50	50	40	75	0	0	75	0	0
p-Phenylenediamine	100	0	0	100	25	0	50	0	400	125	0	0
Caffeic acid	100	100	33	25	25	20	25	0	0	75	0	0
Indoleacetic acid	50	33	67	100	100	80	125	25	100	75	0	100
	In the absence of hydrogen peroxide											
Catechol	0	0	0	0	0	0	0	0	0	0	0	0
Caffeic acid	0	0	0	0	0	0	0	0	0	0	0	0
p-Phenylenediamine	100	0	0	100	25	0	50	0	400	125	0	0

1, 4 and 5 were highly active on eugenol, but did not utilize catechol well. This appears to support the hypothesis that these wall peroxidases could be involved in the synthesis of lignin as a wall component. However, several other enzymes 7, 8, 2, 3 and B also possessed high eugenol activity (Table 5). Considerably high amounts of enzymes 7 and 8 were found in wall fractions but enzymes 2, 3 and B were almost entirely cytoplasmic (Table 3).

Enzymes 1, 2, 3 and 6 used p-phenylenediamine as a substrate without addition of hydrogen peroxide (Table 5), implying that these enzymes could function as laccases. None of the enzymes were able to function as polyphenoloxidase, oxidizing catechol and caffeic acid in the absence of hydrogen peroxide (Table 5). When the gel was incubated with catechol solution for 4 days at room temperature, all peroxidases except enzyme 6 in leaves were lightly stained, to differing extents. This appears due to peroxidative oxidation of catechol, through the gradual generation of peroxide in the system.

Enzymes 1, 2, 3, 4, 5, 7, 8 and B showed broad substrate specificity. Among them enzyme 2 was highly active on all substrates used, followed by enzymes 7 and 3 (Table 5). Enzyme 2 was distinguished by its tissue specificity, detectable only in pollen grains (Table 1). This enzyme may be related to the specific phase of pollen metabolism or sporopollenin production. Enzymes 6, 9, A and C were highly substrate-specific (Table 5). Enzymes 6 and A oxidized only indoleacetic acid and the artificial dyes benzidine and o-dianisidine. This suggests that these enzymes serve only as IAA oxidase or oxidize other unknown natural substrates. Enzyme A is high tissue-specific, detectable only in roots

(Table 1). Enzyme 9 utilized only binzidine and o-dianisidine. This enzyme was distinguished by its high heat-lability (Figure 13).

The ability of peroxidase to catalyze the oxidation of a wide variety of substrates in the presence of hydrogen peroxide has been known for a long time. The existence of peroxidase isozyme has been suggested as an explanation for the broad substrate specificity. Several workers observed different substrate specificities of individual peroxidases by isolated isozymes (McCune 1961; Kawashima and Uritani 1965; Kay et al. 1967) or zymographically (Farkas and Stahmann 1966; Hamill 1970; Liu 1971: Rychter and Lewak 1971; Seevers et al. 1971). From the result presented in Table 5, it appears that many peroxidases have rather broad substrate specificity. Substrate specificities of enzymes may be influenced by pH and other factors such as substrate or enzyme concentration, temperature, activators or inhibitors (Hosoya 1960; Kay et al. 1967). Liu (1971) detected three anodal and three cathodal peroxidase isozymes by benzidine-H_2O_2 staining (pH 8.0) from extracts of horseradish leaf petioles. Two anodal and four cathodal isozymes were detected by eugenol-H_2O_2 staining (pH 6.0). Among the four cathodal isozymes, two of them were negative to benzidine staining. In his experiment the difference of pH seems to have influenced the result rather than substrate specificity. Benzidine was reportedly suitable substrate for many peroxidase isozymes from different sources below pH values of 6.0.

PH Response of Peroxidases

The pH responses of maize peroxidases from 15 day-old mesocotyls were studied (Figure 14). After electrophoresis, peroxidases were

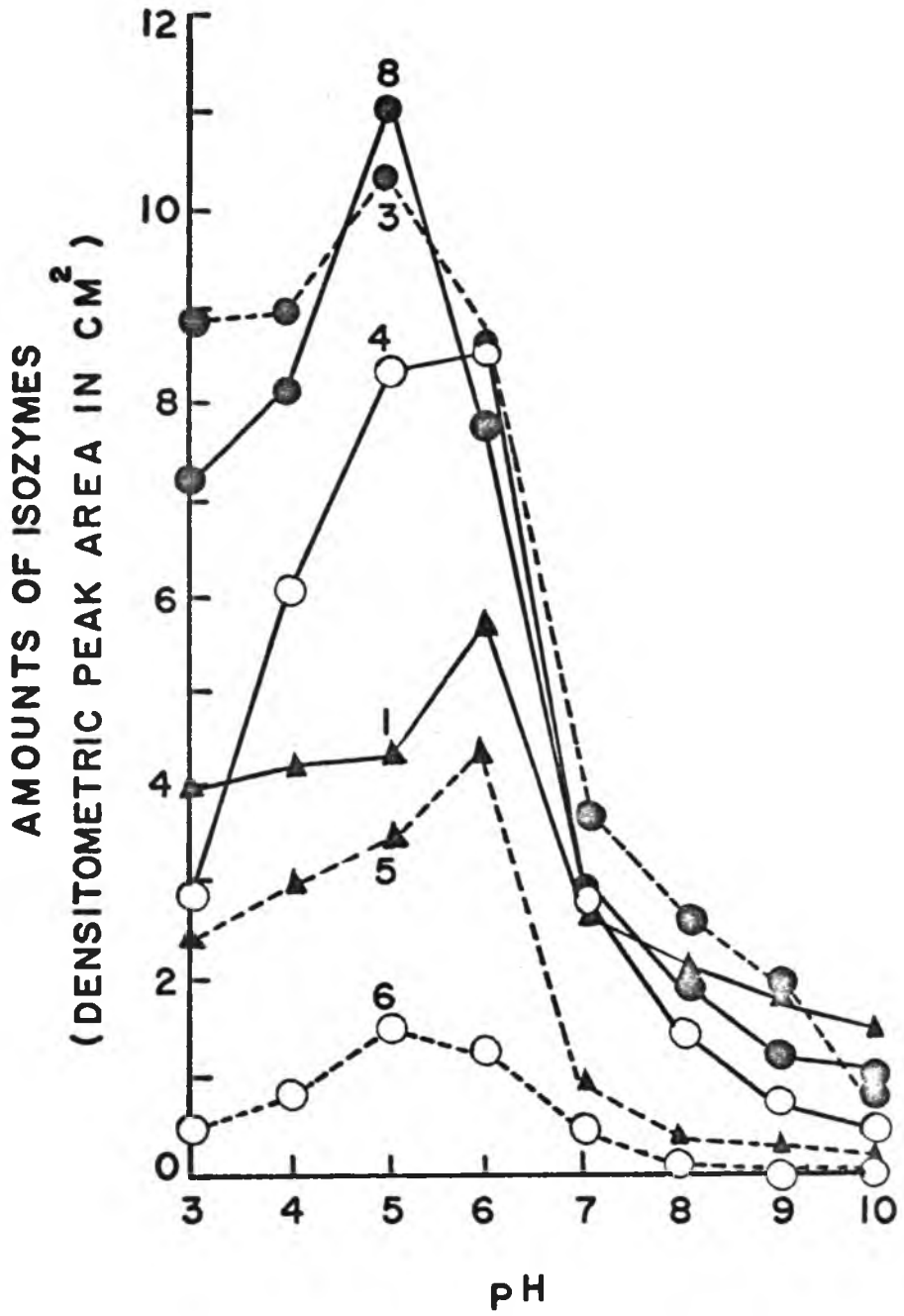


Figure 14. pH optima of peroxidase isozymes in extract of 15 day-old maize mesocotyls.

developed on gels at different pH values between 3 and 10 with o-dianisidine as a substrate. The maize peroxidases appeared to fall into three groups according to their differences in pH optimum. Enzymes 1 and 5 exhibited a sharp pH optimum at 6.0, while enzymes 3 and 8 at 5.0. Enzymes 4 and 6 showed the pH optimum in the region 5.0-6.0 (Figure 14). The entire shape of the pH response curves was not normally distributed, but skewed with a sharp fall above pH values of 7.0 (Figure 14). All enzymes showed high activity at pH values of 4 and 3.

Extracts of the mesocotyls exhibited a sharp pH optimum at 6.0, when assayed colorimetrically and were actually inactive at pH value of 3 (Figure 15). Such a discrepancy of pH optima between isozyme levels on gel and total peroxidase level seems to be due to the interaction between gel components and buffer solution added.

The pH response of peroxidases appear to directly reflect differences of molecular charges of these peroxidases. Anodal enzymes 3 and 8 possessed their pH optimum at a more acidic region than those of cathodal enzymes 1 and 5. Table 14 shows that the region of pH optimum for anodal enzyme 6 is more acidic than that of cathodal enzyme 4, although more critical statistic analysis is required. Kay et al. (1967), by using isolated and highly purified HRP isozymes, demonstrated that optimum pH of anodal isozymes A-1, A-2, and A-3 were 5.8, 5.6, and 5.6, respectively, while that of cathodal isozymes B, C, D, and E were 5.0 on o-dianisidine. Almost identical results were reported by Wang and Dimarco (1972) for purified HRP isozymes.

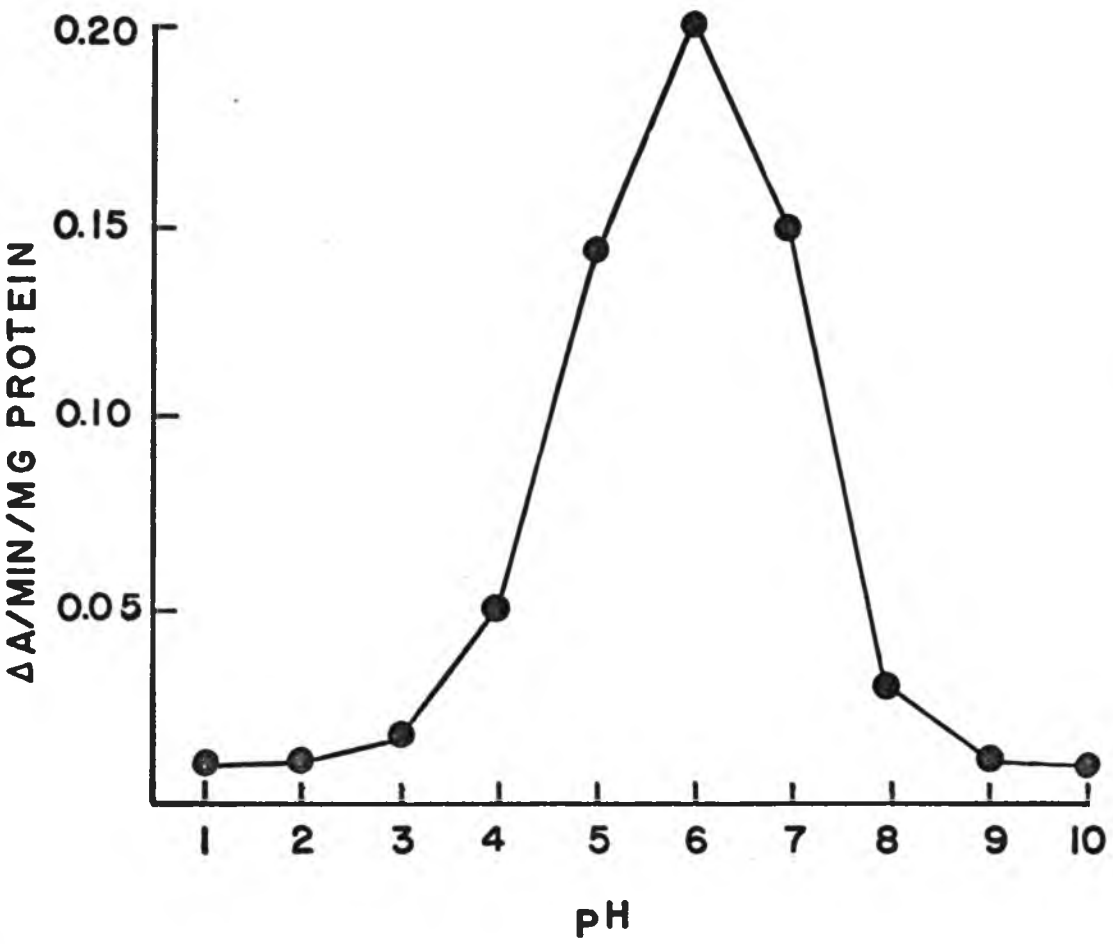


Figure 15. Effect of pH on peroxidase activity of maize mesocotyls of 15 day-old seedlings.

IV. IAA OXIDASE ACTIVITY OF MAIZE PEROXIDASES

Among the many proposed functions of plant peroxidases, the regulation of plant growth via their ability as IAA oxidases is a most significant potential role. Several characteristics of the IAA oxidases from maize leaves were studied, and a method of staining IAA oxidase isozymes on gels was developed for classification of the maize peroxidases.

Peroxidase and IAA Oxidase Activities of Leaf Extracts

Peroxidase and IAA oxidase activities were compared from the extracts of mature corn leaves after treatments with acetone and ammonium sulfate. Leaves were homogenized with 10 times their weight of cold acetone, filtered, and the residue washed with cold acetone and dried. The acetone powder was incubated for 1 hour with 0.02 M phosphate buffer (pH 7.0) to extract peroxidase and filtered. The filtrate was refferred to as acetone powder extract (Table 6). The filtrate was then fractionated with ammonium sulfate (35-75%). The precipitate formed was dissolved with 0.01 M Na acetate buffer (pH 4.5) and dialyzed overnight against the same buffer. After centrifugation at 12,000g for 10 min, the dialysate was used as crude extract for the IAA oxidase study.

The peroxidase activity, protein content, specific activity, and degree of purification of the crude leaf peroxidases are presented in Table 6. The ammonium sulfate fraction had been purified approximately 6.2 fold in comparison to the acetone powder.

Table 6. Peroxidase activity of crude extract of maize

	Enzyme unit (A/min)	Protein content (mg/ml)	Specific activity	Degree of purification
Acetone powder extract	0.154	0.152	1.013	---
Ammonium sulfate extract	0.480	0.076	6.316	6.23

IAA oxidase activity of the crude extract was examined under acidic (0.01 M acetate, pH 4.5) and alkaline (0.02 M phosphate, pH 7.0) conditions. IAA oxidase activity was detected only under acidic conditions (Figure 16). The addition of 10^{-4} M $MnCl_2$ almost doubled enzyme activity, but only at acidic conditions. Even in the presence of manganese ion, no enzyme activity was detected under alkaline conditions. The importance of manganese ion in IAA oxidase action will be discussed later.

Effect of Substrate and Enzyme Concentrations

The rate of IAA destruction varied with IAA concentration from 0 to 80 ug (Figure 17). The amount of IAA destroyed was approximately a linear function of initial IAA concentration at low concentrations. The IAA concentration became non-limiting at concentrations above approximately 30 ug under the experimental conditions (acetate buffer, pH 4.5 and 10^{-4} M $MnCl_2$).

The relation between rate of destruction of IAA and concentration of enzyme is shown in Figure 18. The usual concentration-activity curve was obtained when the enzyme concentration was varied from 7.6 to 76 ug/ml.

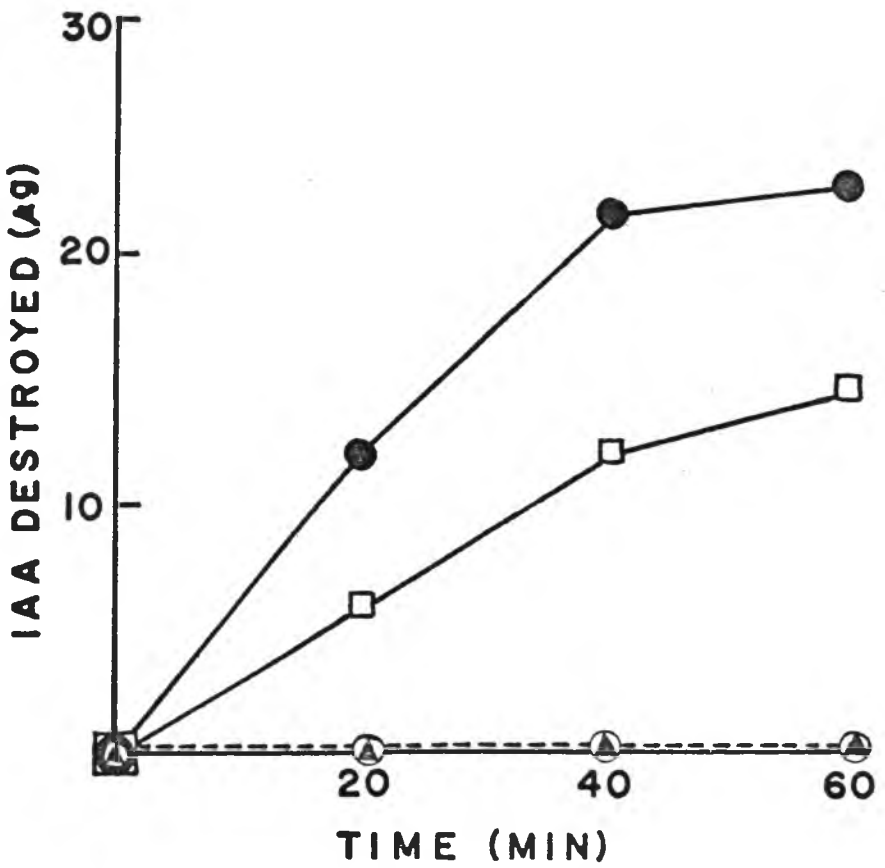


Figure 16. Rate of IAA destruction at different pH in the presence and absence of 10^{-4} M $MnCl_2$.
□—□ pH 4.5
●—● pH 4.5 + $MnCl_2$
▲---▲ pH 7.0
○---○ pH 7.0 + $MnCl_2$

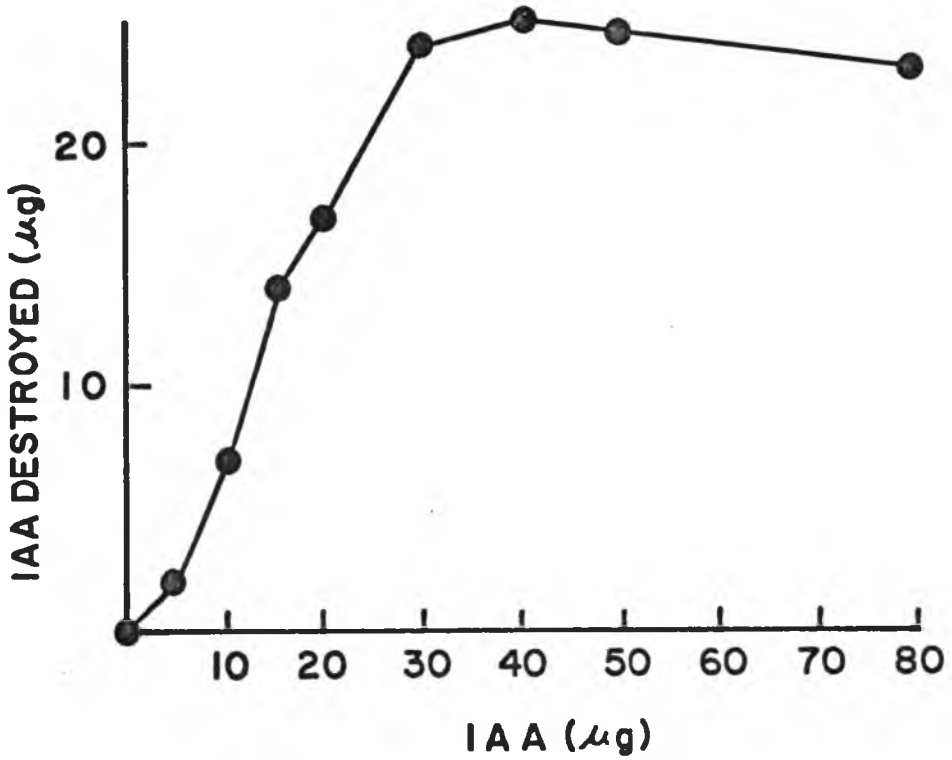


Figure 17. Rate of enzymatic destruction of IAA as a function of substrate concentration.

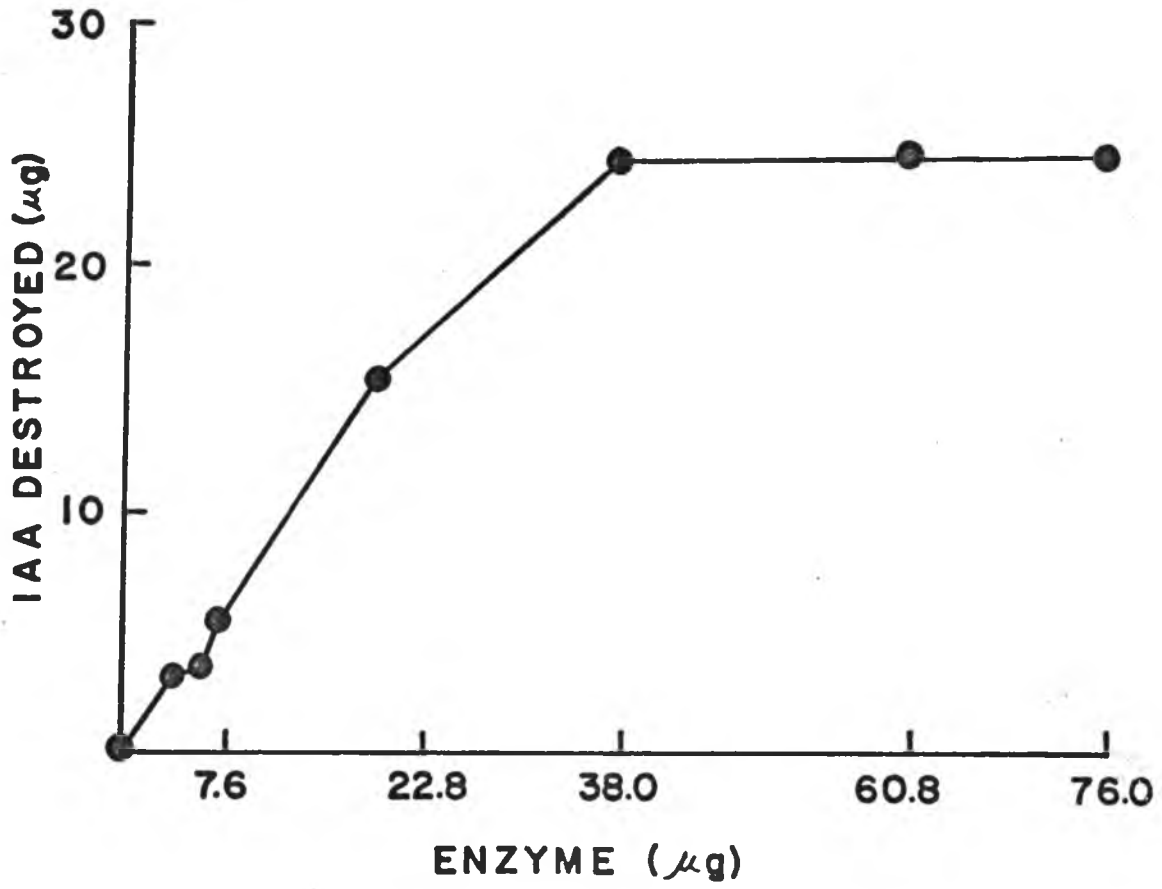


Figure 18. Rate of enzymatic inactivation of IAA as a function of enzyme concentration.

At low enzyme concentrations, rate was proportional to enzyme concentration, while at higher concentrations the substrate became the limiting factor (Figure 17). The reaction was carried out in acetate buffer (pH 4.5) and 10^{-4} M $MnCl_2$.

There is no evidence in these response curves of natural inhibitors, which would be removed by acetone treatment and dialysis. Such natural inhibitors of IAA oxidase have been reported to cause unusual enzyme concentration-activity curves (Tang and Bonner 1947, 1948; Wagenknecht and Burris 1950; Gortner and Kent 1953; Galston and Dalberg 1954; Stutz 1957). The inhibitors were not identified but indirect evidence has shown that they may have been phenolic compounds (Galston and Kent 1953). In any case, the inhibitors were removed by dialysis or further purification of the enzyme. Indole derivatives also can act as peroxidase inhibitors (Ray 1960; Mino 1968).

Effect of pH on IAA Oxidase Activity

Determination of the pH optimum of IAA oxidase activity was made with 0.5 M acetate-HCl buffer (pH 1-6) and 0.2 M phosphate buffer (pH 5-8). In Figure 19 are presented the results of this experiment, showing that the destruction of IAA by the enzyme exhibits a sharp pH optimum in the region 4.0-5.0. This maize leaf IAA oxidase is distinguished by an unusually low pH optimum, and is essentially inactive at pH 7 and above. Optimum activities for IAA destroying enzymes have been reported to lie between 6.2 and 6.7 in peas (Tang and Bonner 1947), between 6.2 and 6.5 in beans (wagenknecht and Burris 1950), about 5.0 in quackgrass (Mudd et al. 1959) or 5.5 in banana (Mace 1967). In contrast, some IAA oxidases have much lower pH optima, e.g., 3.5 in

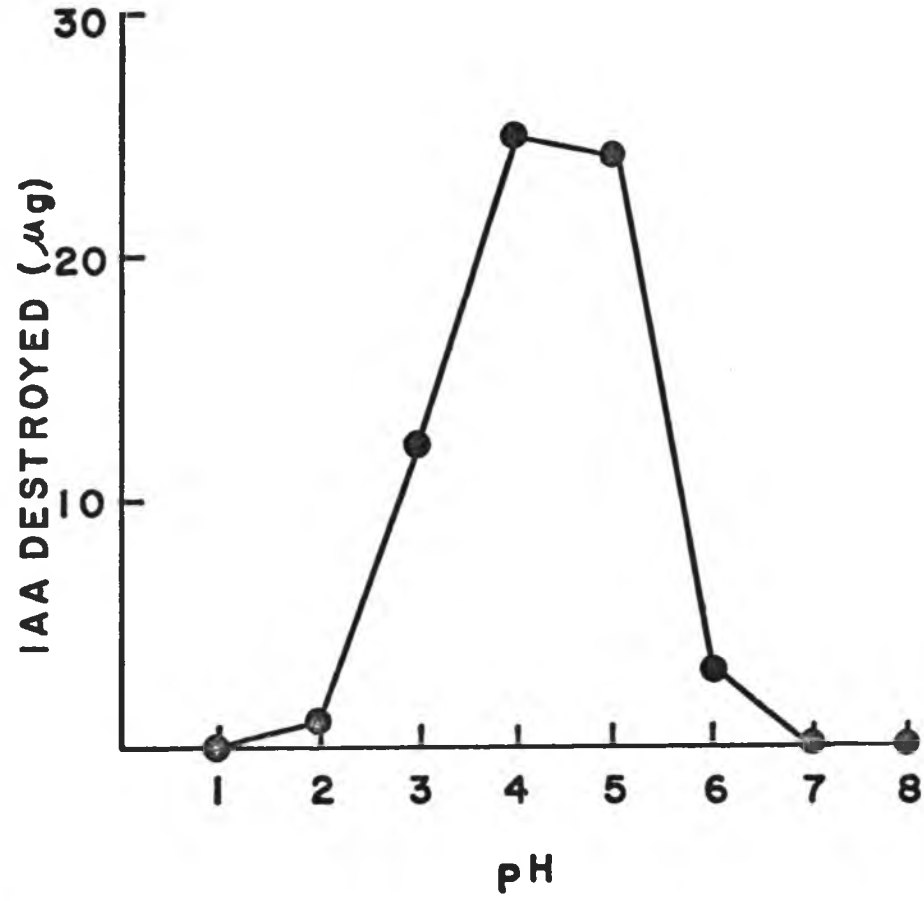


Figure 19. Effect of pH on IAA oxidase activity of maize leaf extract.

pineapple (Gortner and Kent 1953), 3.5-4.0 in common mushroom (Ray and Thimann 1956), 3.5 in horseradish (Hinman and Lang 1965) and 3.6 in Japanese radish (Morita et al. 1962). Much of this inconsistency may stem from the disparity of the sources investigated, degree of purification, and isozymic heterogeneity of the tissues studied.

Data on pH optima are further complicated by the presence of inhibitors or activators. According to Stutz (1957), the crude enzyme extract from lupine showed maximum activity at pH 5.5, but purification shifted the optimum to 6.5, a pH at which the crude extract was inactive. The optimum activity of total IAA oxidase is equivalent to the sum of individual isozyme activities. Since each species or tissue has a different isozyme pattern, properties of whole IAA oxidase may also vary among species or tissues.

Effect of Manganese on IAA Oxidase Activity

It has been reported by several investigators that manganese affected the IAA oxidase activity of several sources in various ways (Gortner and Kent 1953; Kenten 1955; Shin and Nakamura 1962; Taylor et al. 1968; Fowler and Morgan 1972). The effects of varied concentrations (10^{-8} - 10^{-1} M) of Mn^{++} as $MnCl_2$ on IAA destruction was determined with 0.01 M acetate buffer, pH 4.5 at 30 $\pm$ 0.5°C. The oxidase activity at pH 4.5 was stimulated to the greatest extent by 10^{-4} M of $MnCl_2$ and inhibited by higher levels of $MnCl_2$ (Figure 20). These effects of manganese appear to be due to different effect on the enzyme, since boiled enzyme added to mixture of IAA and $MnCl_2$ or blanks (IAA plus $MnCl_2$ with no enzyme added) showed no IAA destruction.

Wagenknecht and Burris (1950) studied the IAA oxidase system of bean

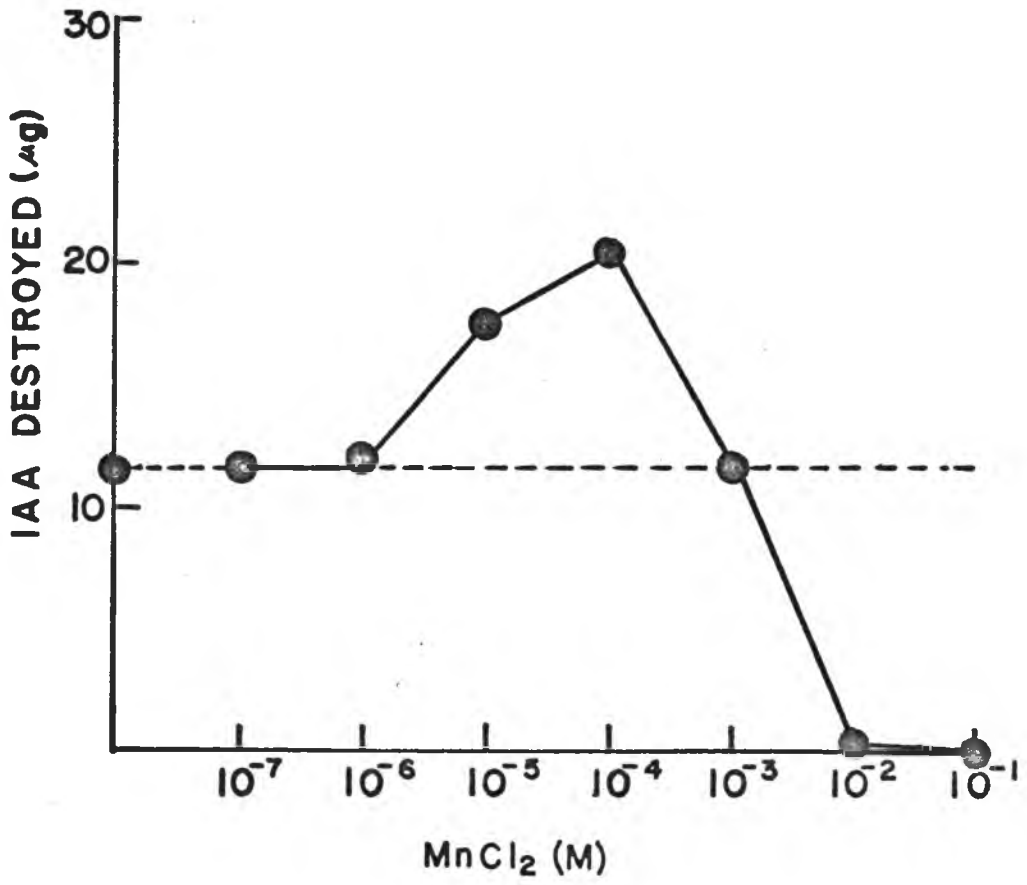


Figure 20. Effect of $MnCl_2$ on IAA oxidase activity of maize leaf extract.

and pea epicotyls. They found that the bean IAA oxidase required Mn^{++} for its activity and that the optimum activity was at 5 x 10^{-3} M of $MnCl_2$. The bean oxidase differed from the pea oxidase, which was inhibited by even lower concentrations of $MnCl_2$ (10^{-5} M). According to Shin and Naka,ura (1962), activity of IAA oxidase from wheat was enhanced greatly at 10^{-5} M of $MnCl_2$ but inhibited at higher concentrations.

The mechanism of manganese involvement in enhancement or inhibition of IAA oxidase activity is unknown. Generation of peroxide by Mn^{++} action seems to be involved in the mechanism. There is some evidence available that manganese ion promotes the generation of peroxide from IAA or other phenolic compounds in vitro experiments (Siegel and Galston 1955). IAA oxidase activity has been reported to be enhanced by low concentrations of peroxide but inhibited by high concentrations (Yamazaki and Souzu 1960). Yonetani and Endo (1969) demonstrated zymographically that IAA oxidase isozymes from several plants respond in different ways to hydrogen peroxide.

High levels of tissue manganese has been reported to increase both peroxidase and IAA oxidase activities, and ethylene-synthesizing systems (Abeles and Rubinstein 1964; Morgan et al. 1966; Yang 1967; Taylor et al. 1968). There are also reports that higher than normal levels of tissue manganese could affect flower differentiation (Joham and Amin 1967; Anderson and Boswell 1968). The activation and inhibition of IAA oxidase activity by manganese suggest that this mechanism may control directly or indirectly several physiological processes in plants.

Effects of Monophenols and Hydrogen Peroxide on IAA Oxidase Activity

It has been reported that substituted monophenols such as

2,4-dichlorophenol (DCP) and 2,4,6-trichlorophenol (TCP) influence IAA oxidase activity (Goldacre et al. 1953), although the mode of action is unknown. Therefore, the effect of a low concentration (10^{-6} M) of TCP on maize leaf IAA oxidase activity with or without 10^{-4} M $MnCl_2$ were examined in 0.01 M acetate buffer, pH 4.5 (Table 7). The oxidase activity was stimulated about 2 fold by TCP, and to the same extent as manganese enhancement (Table 7). However, there was no synergistic effect of TCP and manganese.

In Table 8 are summarized effects of hydrogen peroxide on the IAA oxidase activity in the presence or absence of cofactors 10^{-4} M $MnCl_2$ and DCP. Hydrogen peroxide was inhibitory at high levels (0.05%) in the absence and presence of cofactors, but promoted the oxidase activity at low levels (0.0005%) (Table 8). Manganese and DCP also enhanced the IAA oxidase activity to about two fold in the presence of hydrogen peroxide (0.0005%). Tables 7 and 8 showed that manganese, monophenols and hydrogen peroxide and their combinations enhanced the IAA oxidase activity to about two fold. At least two explanations are possible to explain this. First, levels of substrate was a limiting factor. Secondly, effects of cofactor was compared at their plateau levels.

IAA oxidase activity is reportedly influenced by the presence of DCP or TCP. According to Goldacre (1953), DCP and TCP enhanced IAA oxidase activity in peas at concentrations as low as 10^{-7} to 10^{-5} M, but were inhibitory at high concentrations. The mechanism in which monophenols enhanced IAA oxidase is not known (Tables 7 and 8). If monophenols promote the generation of peroxide in a manner similar to that conjectured for manganese by Siegel and Galston (1955), monophenols

Table 7. Effects of $MnCl_2$ (10^{-4} M) and TCP* (10^{-6} M) on maize leaf IAA oxidase activity

Treatment	IAA destroyed (ug)
Control	12.50
+ $MnCl_2$	20.30
+ TCP	20.30
+ $MnCl_2$ + TCP	19.90

*2,4,6-Trichlorophenol

Table 8. Effects of hydrogen peroxide and cofactors on the maize leaf IAA oxidase activity

	IAA destroyed (ug)
Control	12.5
+ 0.5% H_2O_2	1.3
+ 0.5% H_2O_2 + $MnCl_2$	1.5
+ 0.5% H_2O_2 + DCP*	0.9
+ 0.5% H_2O_2 + $MnCl_1$ + DCP	1.2
+ 0.0005% H_2O_2	24.0
+ 0.0005% H_2O_2 + $MnCl_2$	24.0
+ 0.0005% H_2O_2 + DCP	23.6
+ 0.0005% H_2O_2 + $MnCl_2$ + DCP	24.3

*2,4-Dichlorophenol

might exercise an indirect control over oxidase activity, similar to that proposed for manganese effects.

IAA Oxidase Activity on Gels of Maize Peroxidases

The dual activity of peroxidase as IAA oxidase has been demonstrated on zymograms after electrophoresis through the use of two different staining methods (Endo 1968; Yoneda and Endo 1969; Frenkel 1972). Endo (1968) and Yoneda and Endo (1969) used mixtures of 5 mM IAA, 0.5 nM TCP, 0.2 u acetate buffer (pH 5.0) and 2 mg per ml of Fast Blue BB salt to stain IAA oxidase isozymes on starch gels, obtaining rather uncritical evidence of different activity. Their method is an application of the principle that several diazonium salts such as Fast Blue BB salt couple with some unknown IAA degradation products to form stable precipitates (Seigel 1966). Frenkel (1972) used p-dimethylaminocinnamaldehyde (DMACA) as a coupling reagent with indole rings following acrylamidegel electrophoresis.

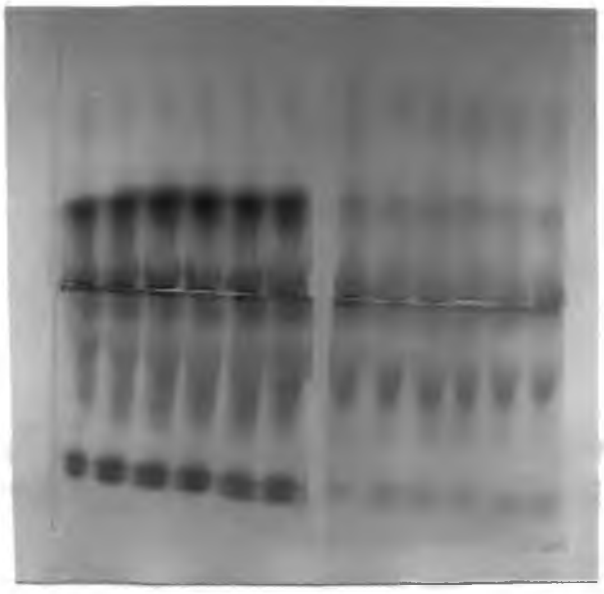
In the present study of maize IAA oxidases on gels, Frenkel's method was first applied. The application of this method resulted in having enzymes 1 and 7 of the crude leaf extract stained as faint purple bands, fading after 1-2 hours. The most prominent peroxidase of leaves, enzyme 3, appeared as a very faint and almost unrecognizable band. The isolated enzyme 3 possessed good IAA oxidase activity. DMACA use has been reported to be non-specific for indole compounds (Bentley 1962). In conclusion, this method is not considered reproducible or reliable enough for the staining of IAA oxidase isozymes.

Endo's method was also examined, but failed to stain any of the isozymes on gels. Price (1972) also failed to detect IAA oxidase

isozymes on starch gels of barley leaf extracts following this method. In principle, Endo's method is better than Frenkel's, because coupled color products are more stable. However, the results seem to be highly variable depending on purity of Fast Blue BB salt (diazotate-4-amino-2,5-diethoxybenzanilide). This compound is prepared by treating cold solutions of aromatic amines in aqueous acid with sodium nitrite, and it degrades easily under light or high temperature (Allinger et al. 1971). If purchased salt contains such degraded impurities, especially phenolics in high amounts, the result is inhibition of IAA oxidase activity.

Several experiments were conducted in an attempt to improve the method of IAA oxidase isozyme staining on acrylamide gels. Several coupling reagents were tested as dyes for IAA oxidases on gels. Following electrophoresis of seedling leaf extracts, gels were incubated in a mixtures of 2 mM IAA, 0.1 mM DCP, 0.1 mM $MnCl_2$, 0.02 M phosphate buffer (pH 6.0) and different coupling reagents, added in this order. The incubation time was 24-48 hours at room temperature in the dark. The coupling reagents included Ehrlich's solution (Meudt and Gaines 1967), Salkowski solution (Meudt and Gaines 1967), and solutions containing 5 mg per ml of Fast Blue BB salt (Sigma, FO250), Fast Blue B salt (Sigma, D3520), Fast Blue RR salt (Sigma, FO500) and Fast Blue BB (K & K, 11606). None of IAA oxidase isozymes were stained by these reagents except Fast Blue BB (K & K Labs, 11601). Accordingly this dye was used for further experiments.

A zymogram of peroxidase and IAA oxidase isozymes from leaf extracts is presented in Figure 21. The extract from 15 day-old leaves were subjected to electrophoresis. The gel was then cut into two sections. Each section was stained individually with o-dianisidine-H_2O_2 and the



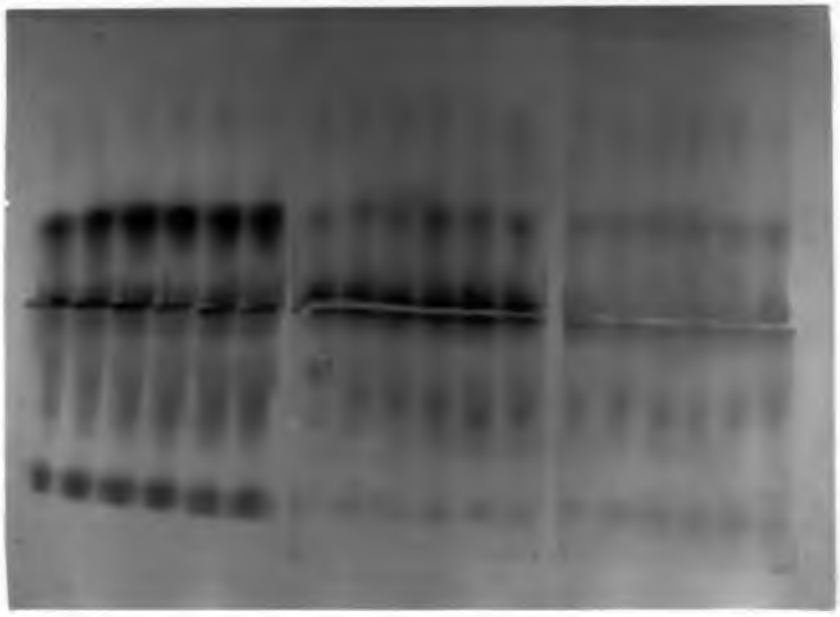
1) Peroxidase 2) IAA oxidase

Figure 21. Zymograms of peroxidase (1) and IAA oxidase (2) in leaf extract of 15 day-old seedlings.

method discussed above employed for IAA oxidase detection. IAA oxidase bands appeared as purplish brown color after overnight incubation. However, further incubation up to 48 hours was necessary for the formation of distinct bands. Except for enzyme 9, all peroxidases investigated possessed IAA oxidase activity (Table 5). No new IAA oxidase bands were observed in addition to peroxidase bands.

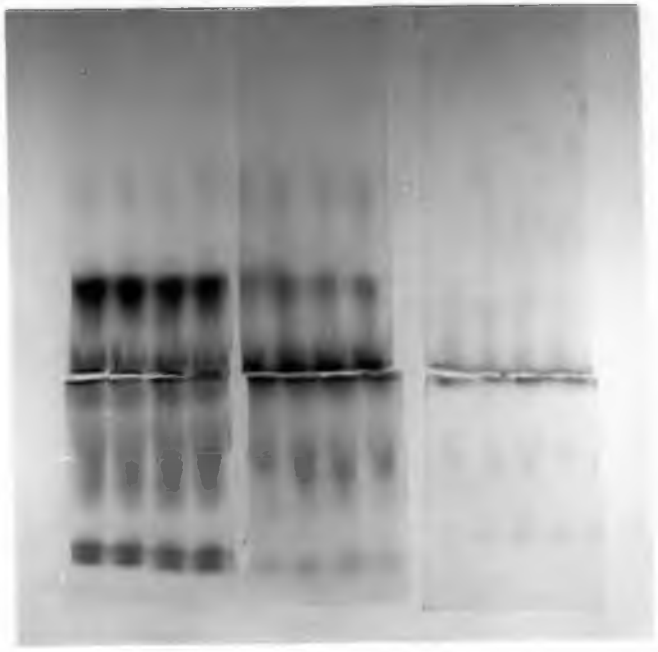
The addition of very low concentrations of hydrogen peroxide to the staining solution for gels also improved staining (Figure 22). In the presence of hydrogen peroxide the incubation time could be reduced considerably without changing the isozyme pattern. Usually it took about 48 hours to get distinct bands without addition of H_2O_2 in the staining solution. However, distinct bands were obtained after 24 hours of incubation if hydrogen peroxide was added. This observation also supports the involvement of peroxide in the IAA oxidase system discussed previously. Peroxide (not necessarily hydrogen peroxide) might be generated during prolonged incubation in the system without addition of hydrogen peroxide. Figure 22 shows the zymogram of IAA oxidase isozymes of leaf extract after 24 hour incubation in the presence and absence of 0.0006% H_2O_2. Higher levels of hydrogen peroxide (0.6%) greatly inhibited IAA oxidase activity (Figure 23).

Yoneda and Endo (1969) reported similar results. Among seven peroxidase isozymes of morning glory callus only 6A and 7A stained as IAA oxidases in overnight incubation without H_2O_2. Addition of low levels of H_2O_2 (0.000015%) stained all isozymes except 4A. Hydrogen peroxide generation seems to be involved also here. If incubation time was prolonged without H_2O_2, isozymes 1A to 5A would be stained except 4A.



1) Peroxidase 2) IAA oxidase ($+ H_2O_2$) 3) IAA oxidase ($+ H_2O_2$)

Figure 22. Zymograms of peroxidase (1) and IAA oxidase stained with (2) and without (3) 0.0006% H_2O_2 after electrophoresis of 15 day-old maize leaf extract.



1) Perox. 2) IAA ox. 3) IAA ox.

Figure 23. Zymograms of peroxidase (1) and IAA oxidase stained with 0.0006% (2) and 0.6% (3) H_2O_2 after electrophoresis of maize leaf extract.

Relationship Between Peroxidases and IAA Oxidases

It appears that the question must be raised whether or not all peroxidases can function as IAA oxidases. As previously observed, all twelve maize peroxidases showed IAA oxidase activity (Table 5), except enzyme 9. This enzyme was extremely heat-labile and unregeneratable (Figure 13). It appeared that this enzyme might be thermally inactivated during incubation at room temperature, resulting in loss of activity of IAA oxidase. Therefore, it seems probable that all peroxidases in maize possess IAA oxidase activity.

According to Yoneda and Endo (1969), all seven peroxidase isozymes of morning glory callus showed IAA oxidase activity except isozyme 4A. The peroxidase activity of this isozyme was presented as a very faint band on their zymogram. This isozyme might be inactivated during overnight staining for IAA oxidase. Although Frenkel (1972) observed that not all peroxidase isozymes in pear and blueberry possessed IAA oxidase activity, the staining method he used appear to be unreliable, as discussed.

All isolated peroxidase isozymes reported so far from various plant sources possessed IAA oxidase activity, except sorghum isozyme A_3 in the report of Stafford and Bravinder-Bree (1972). They demonstrated that the isozyme A_3 isolated from etiolated sorghum mesocotyls had no activity on IAA but five other isozymes did. However, isozyme 3 is implicated in IAA control for at least two reasons. First, this isozyme was detected only in cytoplasmic fraction of cortex, but not in the stele of mesocotyls. Secondly, this isozyme was found in green leaves in addition to mesocotyls but not in roots and ungerminated seeds. Accordingly this

isozyme might have IAA oxidase activity, but they failed to detect it. They used a polarographic method (oxygen cathode) to measure IAA oxidase activity. IAA oxidase and any other oxidase activities can be measured in a few minutes by this method. However, the isozyme A_3 existed in very low activity as peroxidase (ca. 4% of the total activity), and is expected to have an initial lag period before oxidizing IAA (McCune 1961). This situation might make it difficult to estimate the IAA oxidase activity of the isozyme correctly by their method. Thus it seems that all peroxidase isozymes investigated have IAA oxidase activity but their relative amounts are variable according to species, tissues and plant age.

All IAA oxidases studied have also showed peroxidatic activity, except in two cases (Sequeira and Mineo 1966; Van der Mast 1969). Sequeria and Mineo (1966) obtained two main peaks of IAA oxidase from tobacco roots through ammonium sulfate and SE-Sephadex fractionations. One fraction showed peroxidase activity but the other did not. Since their publication, many workers considered that tobacco roots were an exception, proving that peroxidase and IAA oxidase could be different enzymes. However, their conclusion is very doubtful for two reasons. First, they used guaiacol for assays of peroxidases from main and minor peaks (each peak may be an isozyme) after SE-Sephadex column chromatography. That guaiacol is unsuitable for assays of many peroxidases has already been discussed under substrate specificity of peroxidases. Secondly, their first main peak contained large amounts of both IAA oxidase and peroxidase activities, and the second peak demonstrated a high activity of IAA oxidase and very low peroxidase

activity. Accordingly they considered this peak as peroxidase-free IAA oxidase fraction. However, high amounts of peroxidase isozymes do not always possess high amounts of IAA oxidase even if they are due to one enzyme as shown here (Table 5) and by other authors (Macnicol 1966; Kay et al. 1967; Stafford and Bravinder-Bree 1972). The results reported by Van der Mast (1969) can be explained similarly.

Another argument for the identity of peroxidases and IAA oxidases comes from the copper protein theory of IAA oxidase reported between 1950 and 1960. This theory was based on the inhibition of IAA oxidases by copper chelating agents. All of the known plant peroxidases and their isozymes isolated in pure or nearly pure form have been hemoproteins (see literature review). If peroxidase and IAA oxide are due to one enzyme, IAA oxidase must also be a hemoprotein.

The copper protein theory of IAA oxidase is not acceptable for two reasons. First, according to the recent knowledge diethyldithiocarbamate used in the old literatures does not exclusively chelate copper (Dinant and Gasper 1967; Janssen 1970). Secondary, IAA is reportedly destroyed by several enzymes including laccase (Fahraeus and Tullander 1956; Legrand 1957) and polyphenoloxidase (Briggs and Ray 1956; Tomaszewski 1959; Konings 1964) which are supposed to be copper proteins. If the content of these enzymes in crude plant extracts is higher than that of peroxidase (IAA oxidase), the results could be misinterpreted.

In conclusion, it is evident that all maize peroxidases are IAA oxidases. It appears probable that this generalization applies to all plants.

V. EFFECT OF IONIZING RADIATION ON PEROXIDASES AND IAA OXIDASES

In this experiment the relationship between peroxidase and IAA oxidase was further investigated on the basis of their differential response to ionizing radiations by analysis based on specific activity levels and individual isozyme changes.

Effects of Gamma-Irradiation on Plant Growth and Total Oxidase Activities

Figure 24 shows the effect of gamma-irradiation on the growth of mesocotyls in the dark. Dry maize seeds were irradiated at doses between 0 and 500 krads, and the growth of etiolated mesocotyls was measured after 5, 8 and 11 days of growth. The growth rate for all irradiated samples was considerably retarded and actually terminated after 8 days. This effect was more pronounced with increasing dosages.

Peroxidases and IAA oxidases were extracted from these samples and their total activities were measured (Figures 25 and 26). The peroxidase activity in both non-irradiated control and irradiated samples continued to increase throughout the 11-day growth period (Figure 25). The rate of increase was greater in irradiated samples, and all irradiated samples possessed higher peroxidase activity than the control. However, because of irradiation damage (evident in the discoloration and crumbling of tissues) at higher dosages, the peroxidase activity of the sample irradiated with 500 krads decreased at 11 days of growth (Figure 25). The sample irradiated at 100 krads possessed twice the peroxidase activity of sample irradiated at 50 krads. However, peroxidase activities in the samples irradiated at 200 or 500 krads did not show such distinct effects of dosages. It seems that the effect level reached a plateau after 100 krads.

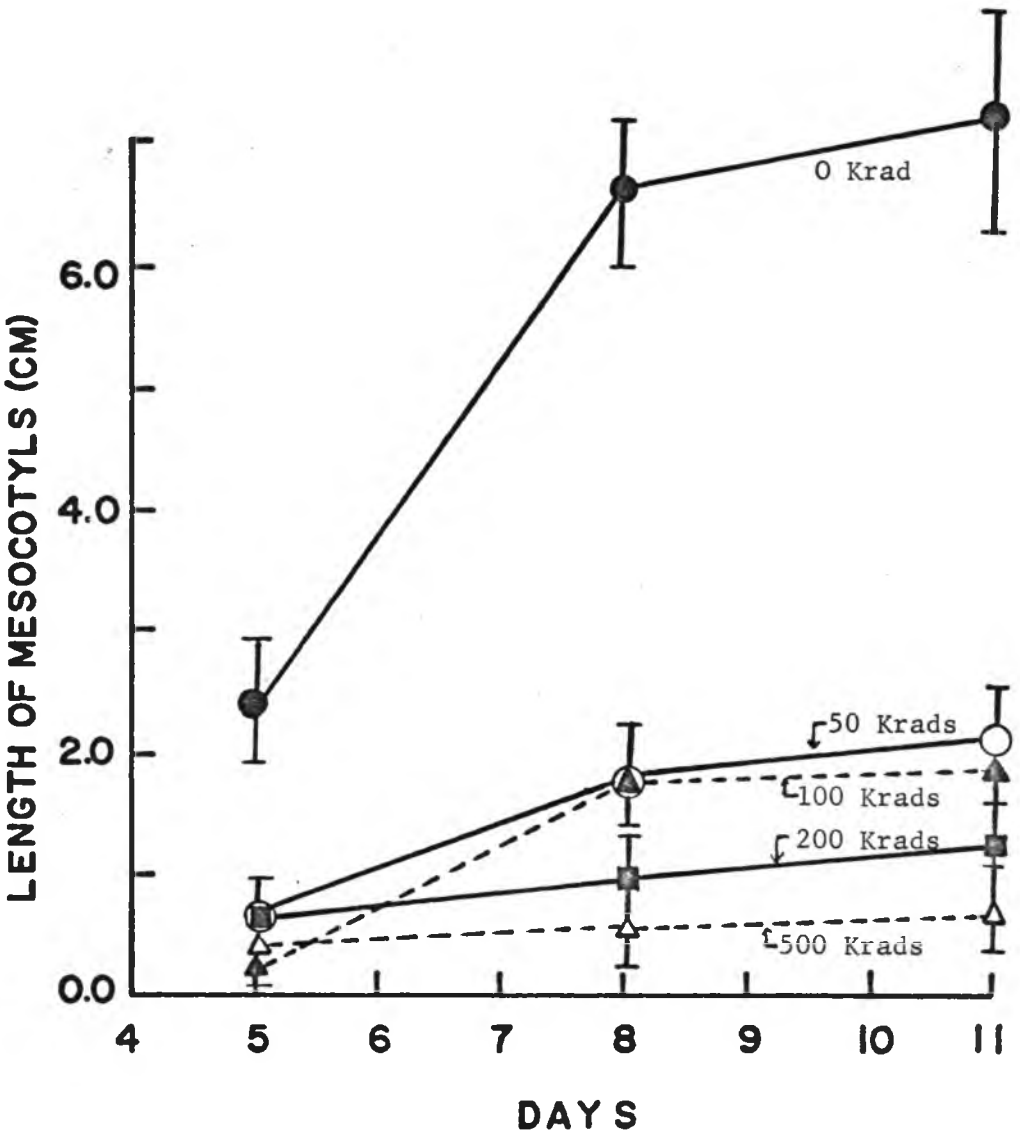


Figure 24. Growth rates of mesocotyls of gamma-irradiated corn seeds between 0 and 500 Krads.

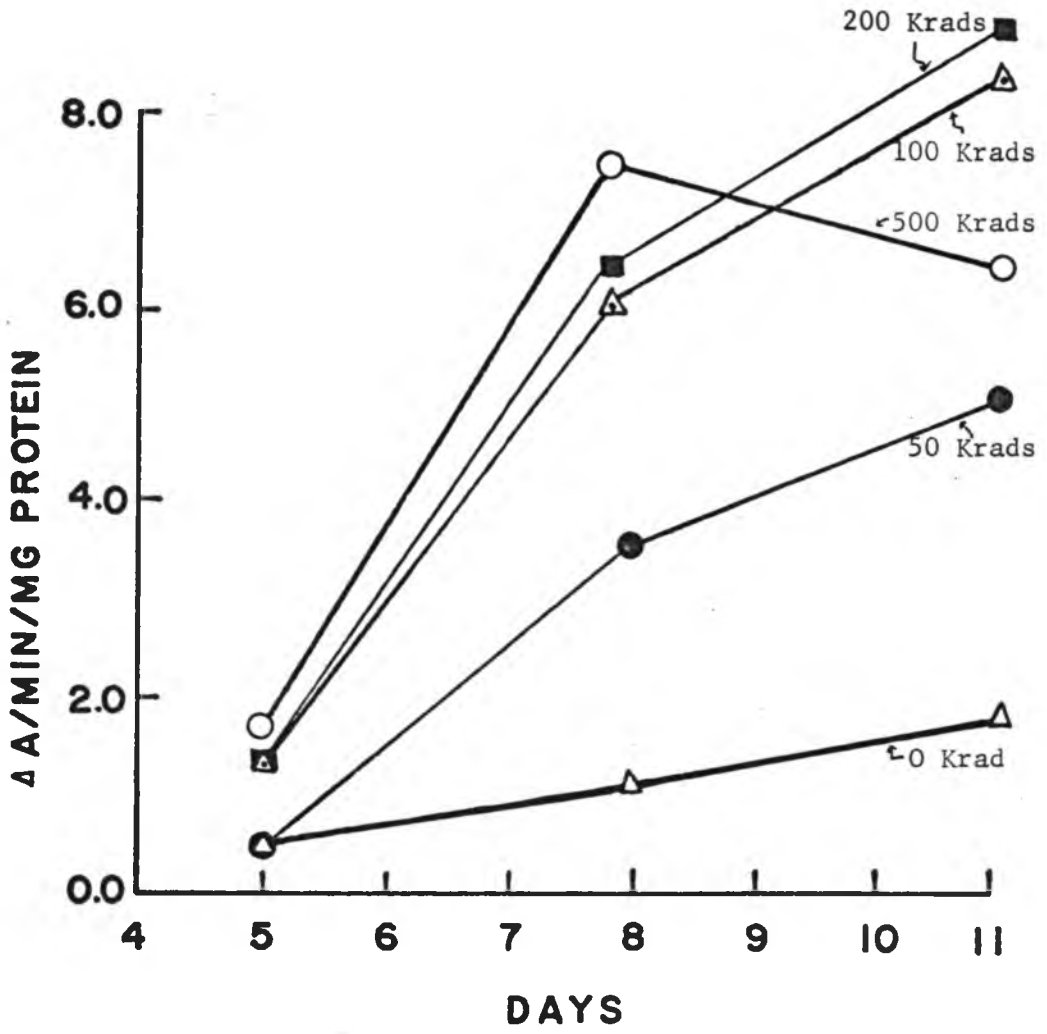


Figure 25. Effect of gamma-irradiation with Cobalt-60 at dose levels of 0 to 500 Krads on the total peroxidase activity of mesocotyls.

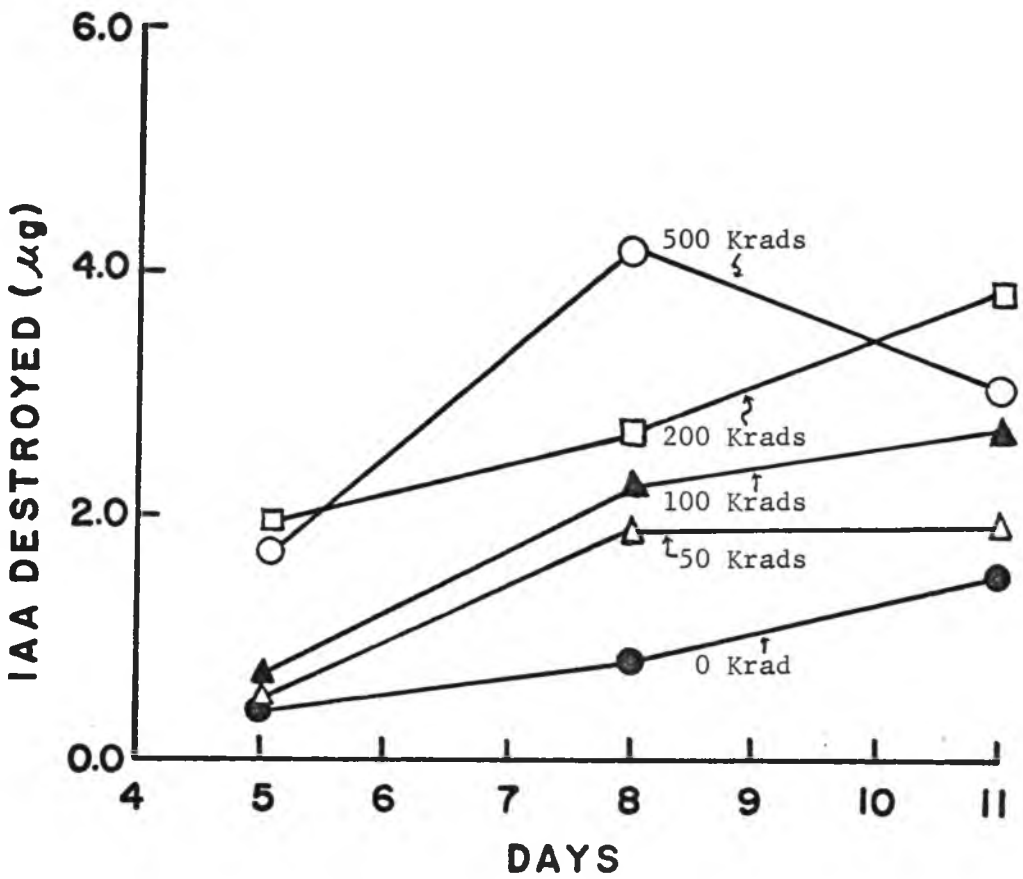


Figure 26. Effect of gamma-irradiation with Cobalt-60 at dose levels of 0 and 500 Krads on the total IAA oxidase activity of mesocotyls.

The IAA oxidase activities showed a similar response to peroxidases following irradiation (Figure 26). They also increased as the plants grew, and were higher at higher dosages. Both peroxidase and IAA oxidase activities increases proportionately after irradiation, generally supporting the argument that these two enzymes are on the same molecule. Several workers reported the activation of peroxidase by ionizing radiation of various types (Endo 1967; Giacomelli 1967; Chourey et al. 1973). Several enzymes in addition to peroxidases, particularly polyphenoloxidases, are activated by low dosages of irradiations (Haskins and Chapman 1956; Fomenko 1970).

Effects of Ionizing Radiation on Isozymes of Peroxidase and IAA Oxidase

Zymograms were prepared of peroxidase and IAA oxidase isozymes from gamma-ray irradiated (0-500 krads) roots, mesocotyls and leaves. In each tissue, radiation increased the intensity of each isozyme of both peroxidase and IAA oxidase. Enzymes 3 and B were increased remarkably by radiation. However, no new bands were formed in response to radiation under these experimental conditions. In Figure 27 are presented the zymograms of peroxidase and IAA oxidase in root extracts from seeds irradiated at different dosages (0-500 krads). In addition to the intensification of bands, (especially enzymes 3 and B), a heavy trailing band appeared from irradiated extracts. The trailing band was less distinct in the extract irradiated with 500 krads, where root growth was considerably retarded. Endo (1967) detected a similar trailing band in gamma-irradiated maize first internodes, calling it a "new band." It is doubtful that this is a new band, but rather due to diffusion caused by the large increase of isozyme concentration. The latter seemed probable

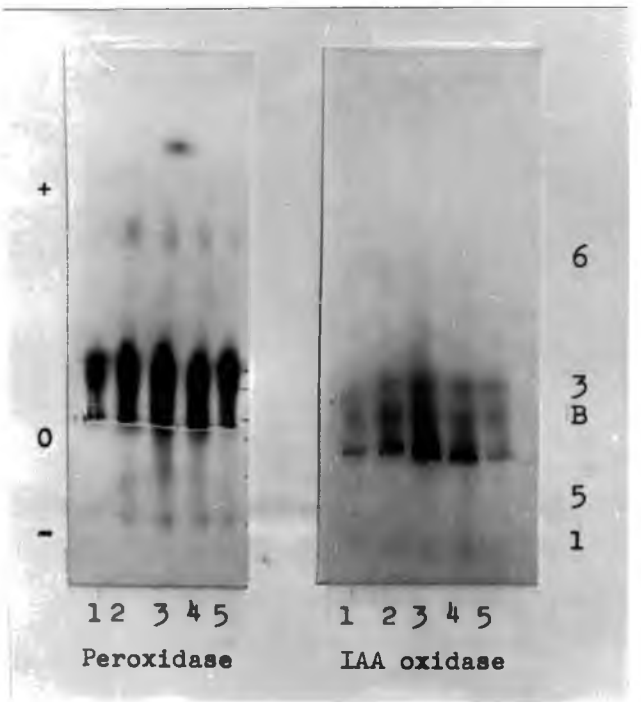


Figure 27. Zymograms of peroxidase and IAA oxidase of roots irradiated with gamma-rays between 0 and 500 krads. Dose levels: 1) 0, 2) 50, 3) 100, 4) 200 and 5) 500 krads, respectively.

because 1) the IAA oxidase zymogram did not contain such a distinct band (Figure 27), and 2) reduction of enzyme concentration applied to the gel reduced such trailing. Both enzyme 3 and B possessed high IAA oxidase activity on the zymogram, but enzymes 1, 5 and 6 showed lower IAA oxidase activity (Figure 27). Zymographically, peroxidase and IAA oxidase behaved similarly (Figure 27).

The Mechanism of Radiation Activation of Peroxidases and IAA Oxidases

In all cases reported here, the peroxidase and IAA oxidase isozymes were activated by relatively low dosages of irradiation. Effects of radiation on the activation of IAA oxidase have not been reported, but that of peroxidase has been often reported (see literature review). Several mechanisms may be involved, some of them specific for peroxidase and others may involve activation of all enzymes. Generally these mechanisms can be classified into four groups: 1) Inactivation of inhibitors, 2) The release of activators, 3) Increased de novo synthesis of the enzyme, and 4) The release of wall-bound or inactive enzymes. In all cases plant hormones may be involved. Fomenko (1970) proposed that the inactivation of unidentified natural inhibitors was more important than de novo synthesis of peroxidases. In contrast, Chourey et al. (1973) explained this by the removal of repression by IAA. Ogawa and Uritani (1970) suggested increased de novo synthesis of peroxidases through enhanced ethylene production.

VI. REGULATION OF PEROXIDASE ACTIVITY BY AN INHIBITORY SUBSTANCE FROM MAIZE POLLEN GRAINS

In the life cycle of a cell, levels of peroxidase activity may be regulated directly or indirectly through many factors. Such factors involve the rate of enzyme synthesis and degradation, availability of substrate, presence of inhibitory metabolites and the microenvironment of the cell. One such possible factor, a water-soluble and low molecular weight peroxidase inhibitor was found in the pollen grains of maize. Several characteristics of the inhibitor were investigated.

Detection of the Inhibitor

Crudely prepared pollen peroxidase was found to be partially inactivated during the course of guaiacol (Figure 28) and o-dianisidine (Figure 29), providing non-linear slopes with time. This inactivation was shown to be caused by a dialyzable substance which was contained in crude pollen extract. Figure 28 and 29 show the changes of peroxidase activity with or without the dialyzable substance. After dialysis, peroxidase activity was affected to a lower degree during the reaction. Addition of the dialyzable fraction to the dialyzed peroxidase greatly inactivated the enzyme (Figure 28 and 29).

Isolation and Partial Purification of the Inhibitor

Pollen grains were ground in a chilled Sorval Omnimix blender for 3 min at high speed with ten times their weight of cold distilled water. The homogenate was dialyzed overnight against the cold distilled water, The dialyzable fraction was then collected and used for further purification. The collected solution was concentrated at room temperature by a vacuum rotary evaporator into one-fiftieth of the original volume. Ten

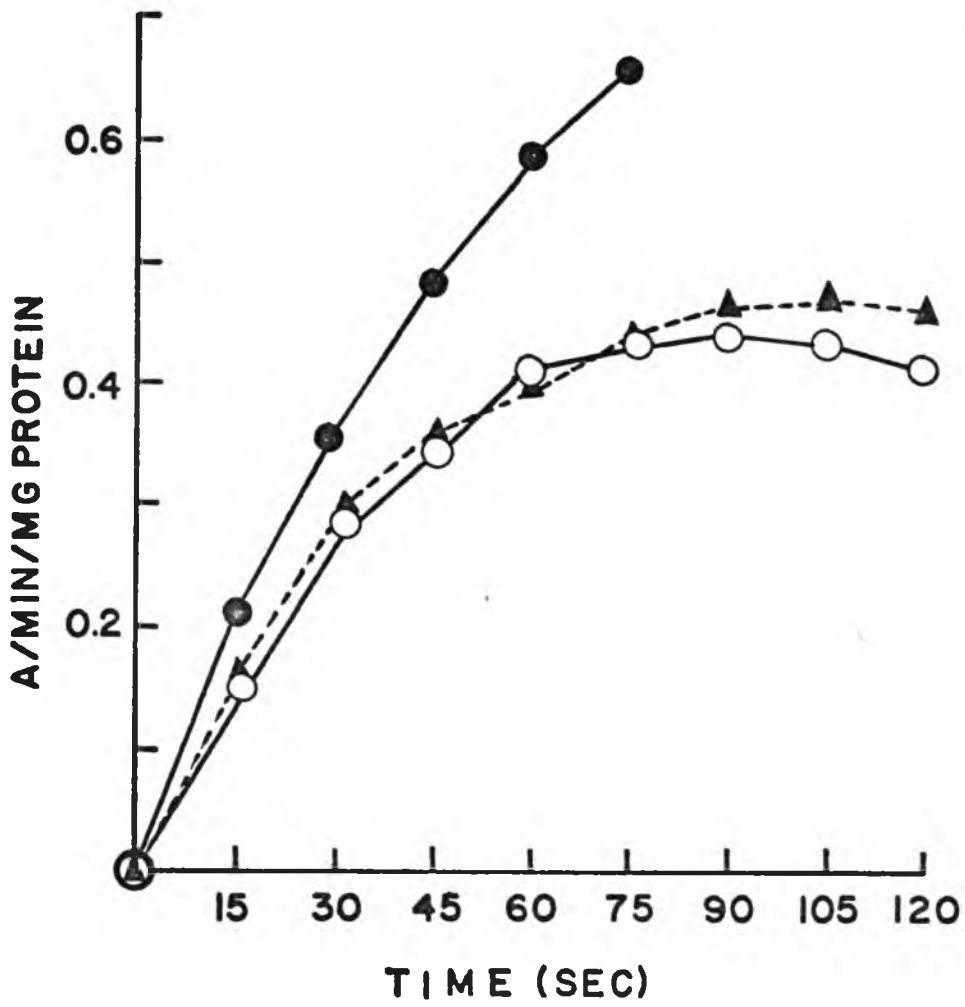


Figure 28. Oxidation of guaiacol by pollen peroxidase.
▲—▲ Non-dialyzed peroxidase
●—● Dialyzed peroxidase
○—○ Dialyzed peroxidase + the dialyzable fraction

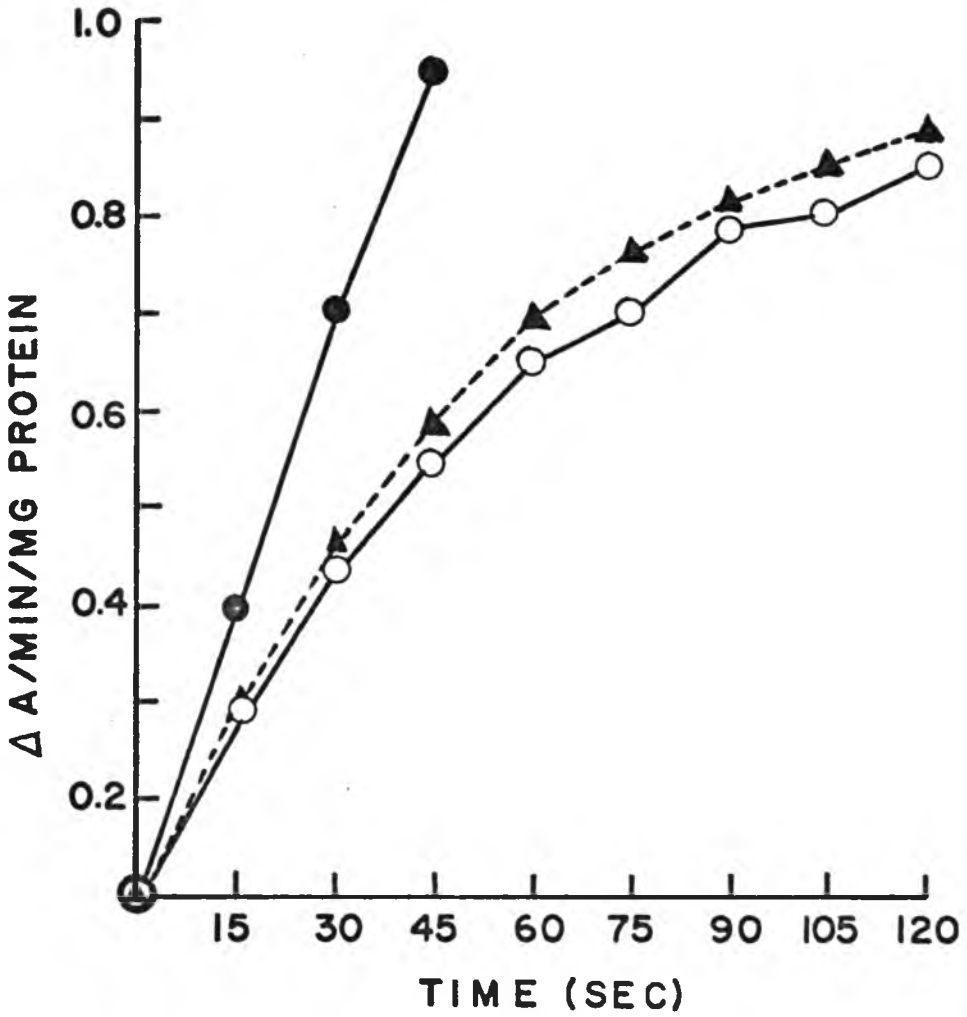


Figure 29. Oxidation of o-dianisidine by pollen peroxidase.
▲—▲ Non-dialyzed peroxidase
●—● Dialyzed peroxidase
○—○ Dialyzed peroxidase + the dialyzable fraction

parts their weight of cold acetone was added to the concentrate. A large proportion of the inactive materials precipitated in the form of a white floc when acetone was added. This was removed by centrifugation (12,000g, 10 min). The inhibitory activity was ascertained to remain in the supernatant. Acetone was evaporated by the vacuum evaporator at room temperature and the inhibitor was extracted with ether from the residue. After the careful removal of ether under vacuum, the residue was dissolved in a small amount of required solvent and used as a partially purified inhibitor.

Effect of pH on the Inhibitory Action

Activity of the pollen peroxidase was assayed in the presence and absence of the inhibitor at ten different pH values (Figure 30). Pollen peroxidase was inactivated to a great extent at lower pH values, especially below pH 4.0. The optimum pH was around pH 6.0, but was shifted to a pH between 5.0 and 6.0 in the presence of the inhibitor. The percentage of inhibition at pH 7.0 was highest (ca. 80% inhibition). This pH value was used in a typical experiment conducted later for the determination of chemical structure and the mode of action of the inhibitor.

Effect of the Inhibitor Concentration

Inhibition of peroxidase was dependent on the concentration of the inhibitor. The partially purified inhibito after ether extraction was dissolved in five ml of distilled water. When increasing amounts of the inhibitor (0-1.0 ml) were added to the reaction system containing peroxidase with ca. 0.375 units, peroxidase activity decreased rapidly to the concentrations of 0.4 ml (Figure 31). The inhibition did not

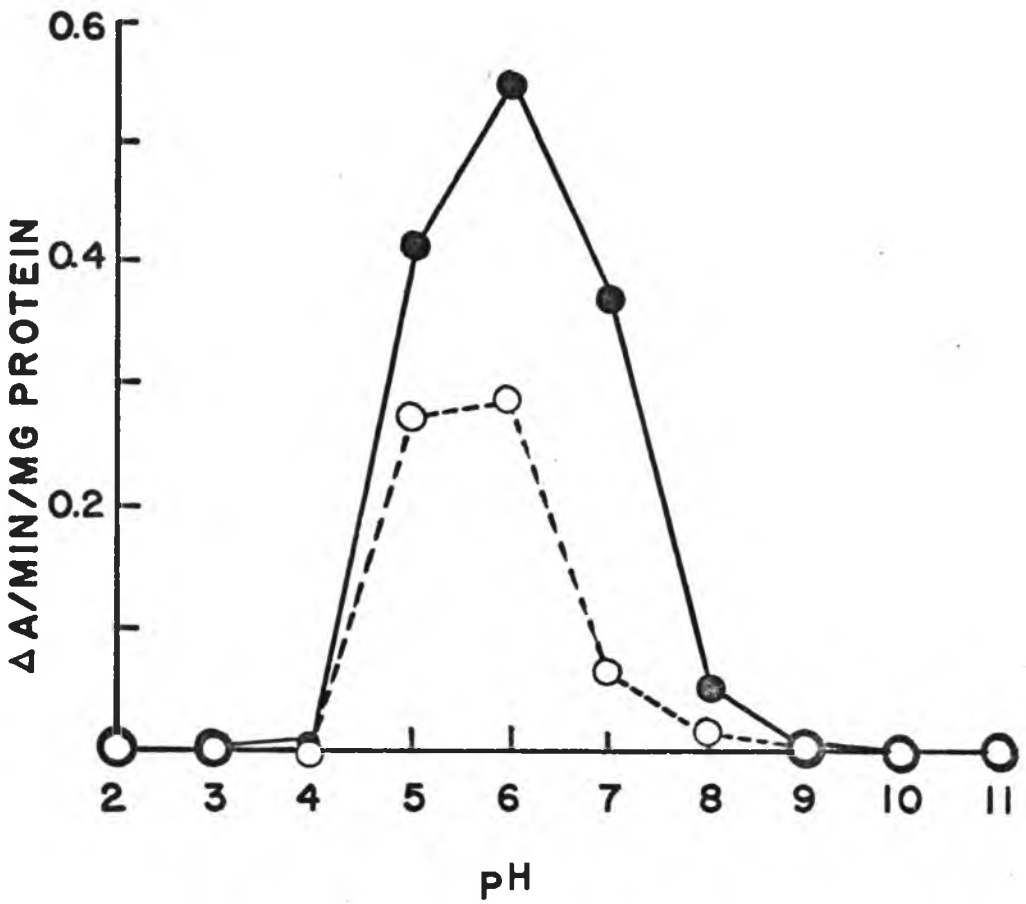


Figure 30. Activity of pollen peroxidase with (o—o) or without (●—●) the inhibitor at different pH values.

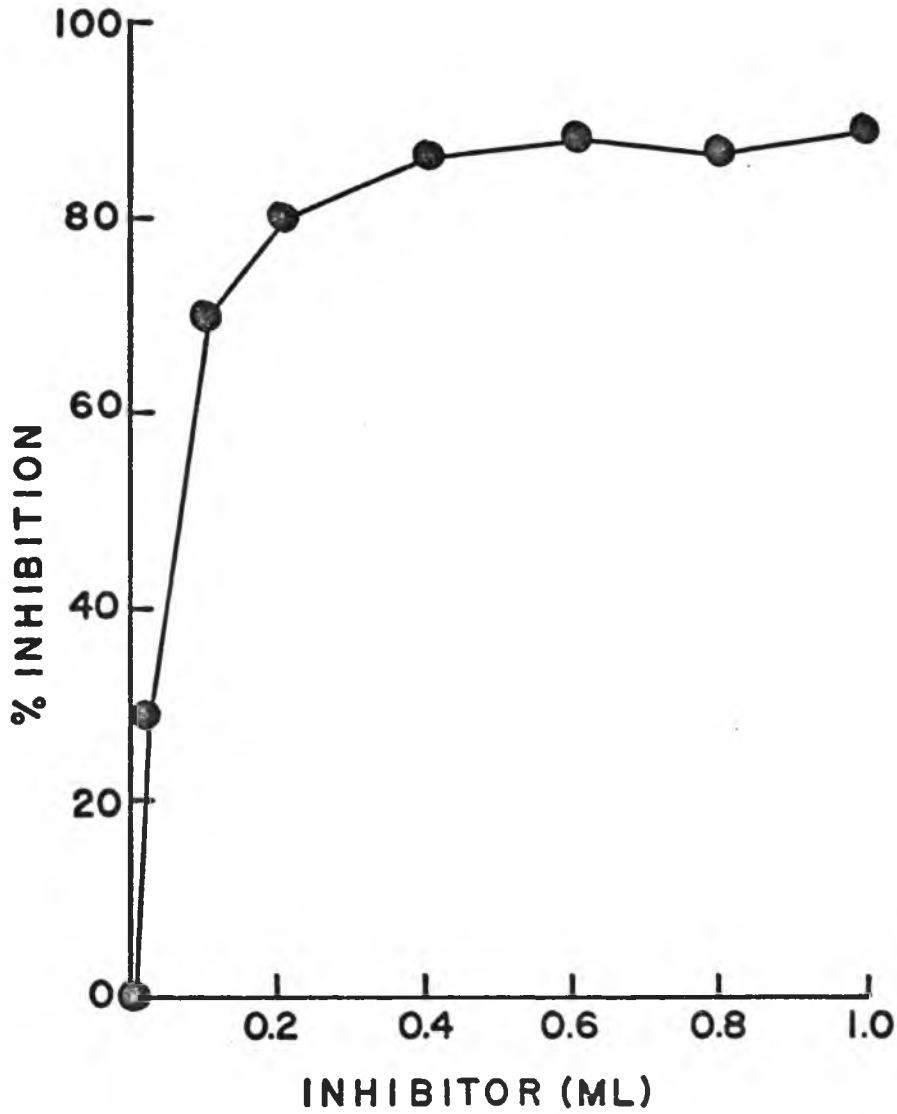


Figure 31. Inhibition of pollen peroxidase activity as a function of amounts of the inhibitor.

reach 100%, as shown in Figure 31. This will be discussed later under the mode of action of the inhibitor. This concentration (o.4 ml) which caused 80% inhibition was used in a typical experiment as a standard.

Effect of Preincubation Time on Inhibition

It was found that the effect of the inhibitor on enzyme action was an immediate response. No latent period of inhibition was observed. Table 9 shows the effect of varied preincubation times of peroxidase with the inhibitor before adding a substrate. Under these experimental conditions the inhibitory action was not influenced by the preincubation time. Immediately after adding the inhibitor to the enzyme reaction mixture, strong inhibition of the enzyme activity was observed (Table 9).

Thermal Stability of the Inhibitor

The effect of heat on the inhibitory activity was examined by heating the inhibitor before addition to the enzyme reaction mixture (Table 10). The inhibitor was heated in boiling water for a period between 0 and 40 min. After cooling the inhibitor, it was added to the peroxidase system and the enzyme activity was compared with the control. The inhibitor was thermally stable and no decrease of inhibitory activity was observed.

Specificity of the Inhibitor

It was found that the pollen inhibitor was not specific for pollen peroxidase but inhibited peroxidases from other maize tissues. Peroxidases were extracted from maize leaves, mesocotyls and roots from 15 day-old seedlings, and pericarp collected from field. The activity of these peroxidases was adjusted to about 0.375 units, and 0.4 ml of the inhibitor was added to the reaction mixture. Approximately 80% of the activity of

Table 9. Effect of preincubation time of peroxidase with the inhibitor on peroxidase inactivation

Preincubation time (min)	Peroxidase activity (A/min/mg protein)
Control	0.372
0	0.075
10	0.075
20	0.074
30	0.075
60	0.077

Table 10. Thermal stability of the inhibitor

Heating time (min)	Peroxidase activity (A/min/mg protein)
Control	0.372
0	0.076
5	0.076
10	0.072
20	0.080
40	0.076

Table 11. Effect of the inhibitor on peroxidase activity from different maize tissues

	Peroxidase activity (A/min/mg protein)	
Enzyme source	No inhibitor	Inhibitor added
Pollen	0.375	0.075
Leaf	0.410	0.095
Mesocotyl	0.355	0.073
Root	0.359	0.071
Pericarp	0.300	0.061

these peroxidase was inhibited by the addition of the inhibitor (Table 11).

The pollen-derived inhibitor was also found to inhibit the action of polyphenoloxidase (catecholase) of maize (Figure 32). Catecholase was extracted from mature maize leaves with distilled water and incubated with the inhibitor. Catecholase activity was inhibited considerably by the addition of the inhibitor. No color development was observed when catechol solution alone was left to stand at room temperature for 2 or 3 hours. However, color development was observed when catechol and the inhibitor were incubated together without adding the enzyme. This will be further discussed in conjunction with the mode of action of the inhibitor.

Chemical Properties of the Inhibitor

To determine the chemical structure of the partially purified inhibitor, several qualitative chemical tests were conducted according to the methods of Shriner and Fuson (1940). The chemical tests involved alcoholic silver nitrate reaction, Tollens' reaction, Fehling's reaction, 2,4-dinitrophenylhydrazine test and ferric chloride reaction.

The inhibitor showed positive reactions to Tollens' reagent, Fehling's reaction and 2,4-dinitrophenylhydrazine. This suggests the possible involvement of an aldehyde group in the inhibitor molecule. The inhibitor also reacted positively to the indole group test through the use of Erhlich reagent and dimethylaminocinnamaldehyde tests (Bentley 1962).

Since the partially purified inhibitors appeared to contain indole and aldehyde groups, several possibilities were considered. Indole-3-aldehyde and indole-3-acetaldehyde was taken into account initially as

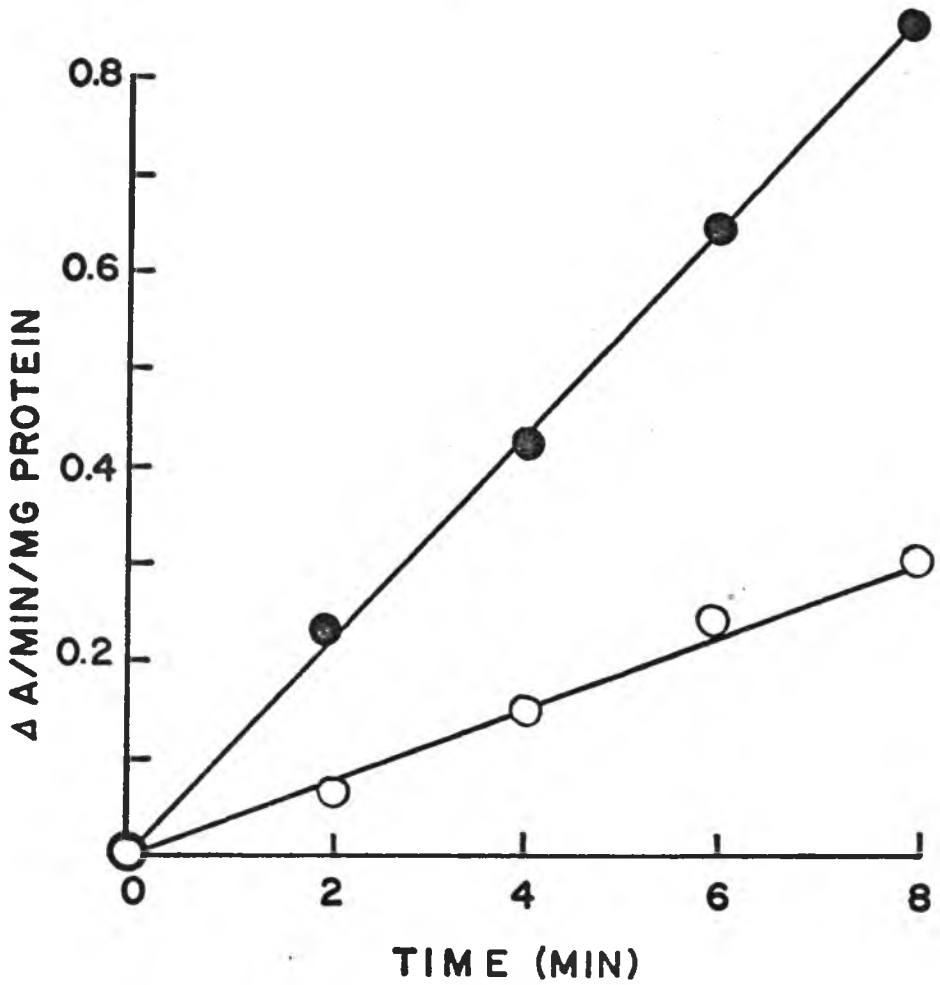


Figure 32. Activity of catecholase from maize leaf with (O—O) or without (●—●) the inhibitor.

the most simplified candidates. The absorption spectrum and Rf value of the inhibitor was compared with those of indole-3-aldehyde and indole-3-acetaldehyde. The ultraviolet absorption spectrum of the inhibitor in ethanol showed absorption maxima at wavelengths of 222, 260, 270 and 289 nm (Figure 33). This did not correspond with the absorption maxima values reported for indole-3-aldehyde (Racusen 1955) and indole-3-acetaldehyde (Gray 1959; Larsen and Klungsoyr 1964; Larsen 1966).

After paper chromatography of the inhibitor, the paper chromatogram was sprayed with Ehrlich reagent or dimethylaminocinnamaldehyde solution to detect indole compounds. The inhibitor was spotted as a violet spot with an Rf value of around 0.24. The Rf values of the indole-3-aldehyde and indole-3-acetaldehyde which were co-chromatographed with the inhibitor were 0.89 and 0.75, respectively. In addition to the main spots, several other spots were observed from the commercially purchased indole compounds. The main spots and minor spots did not correspond with the inhibitor spot. When the chromatogram of the inhibitor was monitored under the UV light with short-wavelengths, several spots were detected in addition to the indole spots. The spots were cut from the paper and eluted with 95% ethanol. The inhibitory activity of the each spot was examined. Only the spot with Rf value of 0.24 had strong inhibitory activity.

It was found that this spot at Rf 0.24 consisted of an indole compound and an unknown non-indole compound. The spot was treated with hydrochloric acid adjusted to a pH value of 2.0. Then the spot was chromatographed and monitored. The monitoring of the chromatogram revealed that there were two spots from the acid-treated spot. One spot had an Rf value of about 0.10 and was negative to the indole test. The other spot had an Rf value around 0.90 and showed a positive reaction to

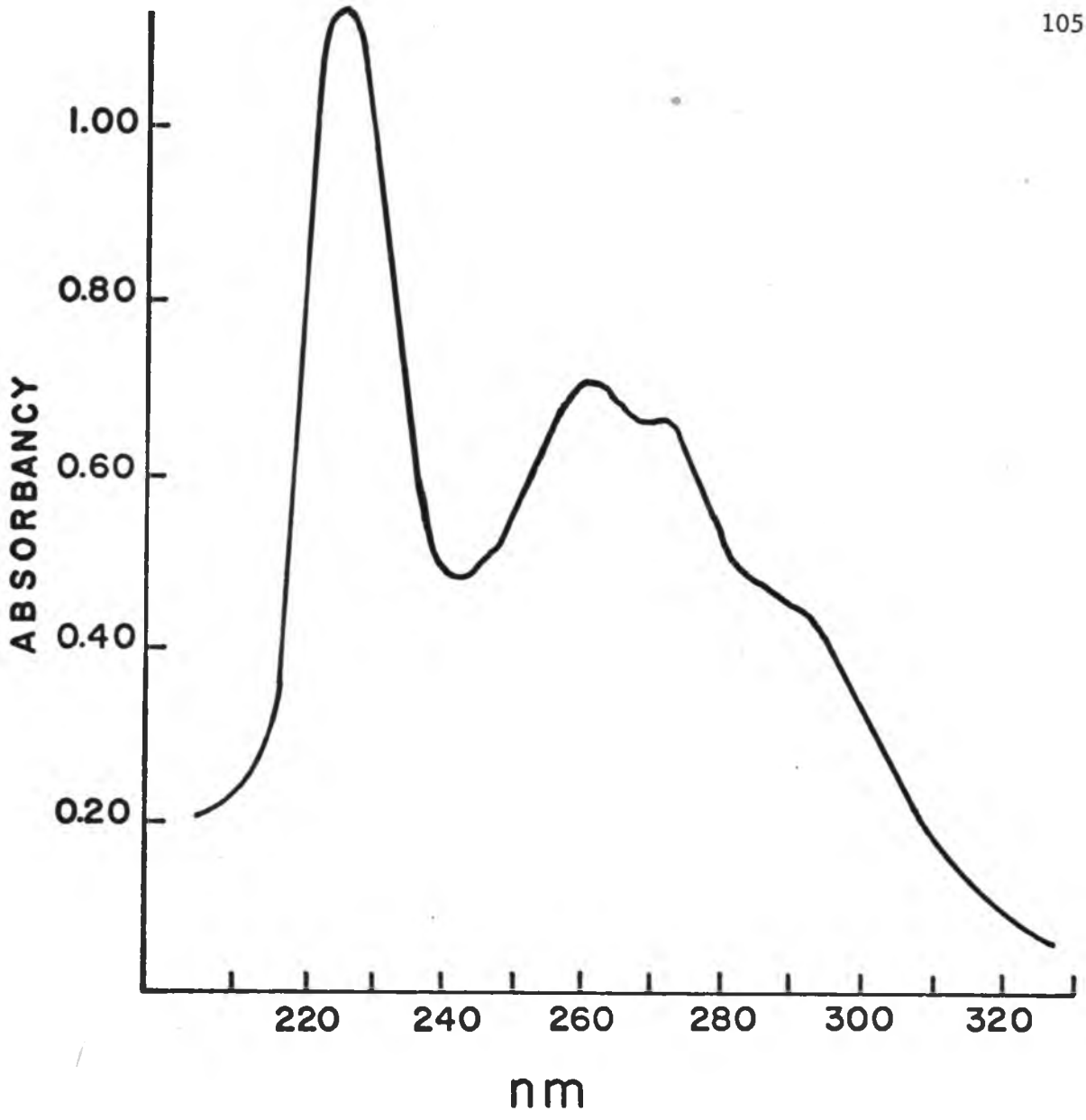


Figure 33. The ultraviolet spectrum of partially purified inhibitor from pollen grains in ethanol solution.

the indole test. This Rf value was very similar to that of indole-3-aldehyde.

No definitive conclusion can be made from the results. However, it seemed that the inhibitor might be a compound of indole-3-aldehyde with an unknown compound.

Mode of Action of the Inhibitor

The catecholase-inhibitor system was first selected to examine the possible mode of action of the inhibitor. The catecholase reaction mixture was incubated for 30 min with or without the inhibitor. In addition to the two systems, catechol and the inhibitor without enzyme was also incubated for 30 min. As discussed previously, a color development was observed when catechol and the inhibitor were incubated. The visible absorption spectra of the three reaction mixtures were compared. The colored product from the enzyme-catechol reaction mixture showed the maximum absorption peak it around 420 nm. However, the colored product from the enzyme-catechol-inhibitor system showed a maximum at around 530 nm and the peak at 420 nm was absent. The same maximum peak (530 nm) was observed when the colored product from the catechol-inhibitor mixture was examined.

Similar results were obtained from the peroxidase system. The reaction product of the peroxidase-0-dianisidine-H_2O_2 system had a maximum peak at 460 nm. In contrast, the maximum peak was at 580 nm when the peroxidase-o-dianisidine-H_2O_2-inhibitor system or o-dianisidine-H_2O_2-inhibitor system was examined.

More detailed research is required to draw any conclusion on the mode of action of the inhibitor. Several possibilities were considered. The inhibitor seemed to compete with the enzyme substrate, resulting in the

inhibition of the enzyme. The inhibitor also seemed to combine with an enzyme to inhibit the reaction since 0.05% albumin reduced inhibition when added to the peroxidase-o-dianisidine-H_2O_2-inhibitor system.

Future Studies of the Nature of the Inhibitor

The *in vitro* enzymatic studies or paper chromatography of extracts from maize leaves revealed that the inhibitor was not likely to be present in them. It is difficult to relate the action of such an inhibitor to pollen physiology, although it might play roles in the ephemeral nature of maize pollen viability, and in their complete loss of viability during freezing or dessication. The determination of chemical structure and chemical synthesis of the inhibitor is necessary for a better understanding of its action or metabolic roles.

The inhibitor might be distributed not only in pollen grains but also among various tissues. In such tissues the inhibitor might play an important role in controlling auxin metabolism. More research concerning the distribution of the inhibitor among different tissues of maize or among various plants is suggested.

SUMMARY

Intracellular localization and biochemical properties of twelve major peroxidases were studied in different tissues of maize. After separation of cytoplasmic fractions by treating each tissue with distilled water, the residual wall pellet was incubated with calcium ion. It was found that 0.2 M $CaCl_2$ facilitated the release of peroxidases from the isolated cell walls up to 60 minute-incubation. A high proportion (ca. 30%) of peroxidase activity was associated with cell wall fractions. Six to ten percent of the wall peroxidase activity was found to be Ca^{++}-insoluble, covalently-bound.

The 12 major peroxidases of maize from cytoplasmic and wall-bound (Ca^{++}-soluble) fractions were quantitatively analyzed by densitometric methods after electrophoresis on 7% acrylamide gels (pH 8.1). Anodal peroxidases were mainly cytoplasmic; those with Rf values of more than 25 were highly associated with the cytoplasmic fractions. Cathodal peroxidases were highly wall-bound. Anodal peroxidase 8 and non-migrating peroxidase 7 were found in equal amounts in both cytoplasmic and wall-bound fractions.

The anodal paroxidases were highly diversified and appeared to be involved in broad physiological processes such as growth regulation, coleoptilar function, photosynthesis or nutrient absorption. Most of them were rather low substrate-specific. However, peroxidases 6, A and C oxidized only IAA among naturally-occurring substrates, suggesting highly specified roles of these peroxidases.

The cathodal peroxidases were largely associated with the wall fractions, evidentally related to a role of lignification in cell walls.

They became increasingly wall-bound during ontogeny. They accounted for about 30% of the total peroxidase activity in normal plants and for only 16% in the low lignin mutant genotype, bm_3, and in the brittle-stalk mutant, bk_2. The substrate specificity studies revealed that they were highly active on eugenol, a presumed lignin precursor. The cathodal peroxidases also showed broad substrate specificity, suggesting multiple functions in addition to lignification.

The substrate specificity studied showed that benzidine and o-dianisidine were good substrates for staining all maize peroxidases and produced identical results for the 12 peroxidases. Pyrogallol and guaiacol were not suitable substrates for visualization of all maize peroxidases, and staining was slow on each. None of the peroxidases were able to oxidize catechol and caffeic acid in the absence of hydrogen peroxide. Peroxidases 1, 2, 3 and 6 (in roots) showed laccase activity when p-phenylenediamine was used as a substrate.

IAA oxidase activity characterized all maize peroxidases. Cofactors such as manganese and monophenols enhanced IAA oxidase activity. The addition of very low amounts of hydrogen peroxide facilitated IAA oxidation without cofactors. IAA oxidase isozymes were stained on gels by using diazonium salt (Fast Blue BB) as a coupling reagent. All peroxidases were found to possess IAA oxidase activity through the staining method. No new IAA oxidase bands were observed in addition to peroxidase bands.

Activities of total soluble peroxidase and IAA oxidases increased after gamma-irradiation of 0 to 500 krads. All peroxidase and IAA oxidase isozymes were intensified similarly by irradiation. The

irradiation did not cause the formation of new isozymes of either peroxidase or IAA oxidase.

A peroxidase inhibitor extracted from maize pollen grains was described as a possible regulator of plant metabolism through the control of peroxidase activity. The inhibitor was partially purified though acetone and ether fractionation. It was water-soluble and thermally-stable, and contained aldehyde and indole groups. Paper chromatography, after acid hydrolysis of the inhibitor, suggested that the inhibitor was a complex of indolealdehyde-like compound and an unknown non-indole compound.

LITERATURE CITED

Abeles, F. B. 1972. Biosynthesis and mechanism of action of ethylene. Ann. Rev. Plant Physiol. 23:259-292.

Abeles, F. B. 1973. Ethylene in Plant Biology. Academic Press, N. Y.

Abeles, F. B. and B. Rubinstein. 1964. Cell-free ethylene evolution from etiolated pea seedlings. Biochim. Biophys. Acta 93:675-677.

Allinger, N. L., M. P. Cava, D. C. De Jongh, C. R. Johnson, N. A. Lebel and C. L. Stevens. 1971. Organic Chemistry. Worth Publishers, Inc., N. Y.

Alvarez, M. R. 1968. Temporal and spatial changes in peroxidase activity during fruit development in Encyclia tampensis (Orchidaceae). Amer. Jour. Bot. 55:619-625.

Anderson, O. E. and F. C. Boswell. 1968. Boron and manganese effects on cotton yield, lint quality and earliness of harvest. Agronomy Jour. 60:488-493.

Azhar, S. and C. R. K. Murti. 1971. Effect of indole-3-acetic acid on the synthesis of cyclic 3-5 adenosine phosphate by bengal gram seeds. Biochem. Biophys. Res. Comm. 43:58-64.

Bach, A. and B. Wilensky. 1930. Zur Kenntnis der Thermolabilitat der Fermente. I. Mitteilung: Uber das Verhalten von durch Ultrafiltration gereinigten Peroxydaseertrakten gegen Siedehitze. Biochem. Z. 226:482-488.

Bailey, L. F. and J. S. McHargue. 1943. Enzyme activity in tomato fruits and leaves at different stages of development. Amer. Jour. Bot. 30:763-766.

Barman, T. E. 1969. Enzyme Handbook. Springer-Verlag, N. Y.

Barton, D. H. R., A. M. Deflorin and O. E. Edwards. 1956. The synthesis of usnic acid. Jour. Chem. Soc. 530-534.

Bentley, J. A. 1962. Analysis of plant hormones. In: D. Glick ed., Methods of Biochemical Analysis, Vol. 9. pp. 75-125. Interscience Publishers, N. Y.

Bingham, W. E., S. L. Krugman and E. F. Estermann. 1964. Acrylamide electrophoresis of pine pollen proteins. Nature 202:923-924.

Birecka, H. and A. W. Galston. 1970. Peroxidase ontogeny in a dwarf pea stem as affected by gibberellin and decapitation. Jour. Exp. Bot. 21:735-745.

Birecka, H. and A. Miller. 1974. Cell wall and protoplast isoperoxidases in relation to injury, indoleacetic acid, and ethylene effects. Plant Physiol. 53:569-574.

Brewbaker, J. L. and D. E. Hamill. 1972. The maize peroxidases; designation of seven loci govering peroxidase polymorphisms in maize. Maize Genetics Cooperative Newsletter 46:29-33.

Brewbaker, J. L. and Y. Hasegawa. 1974a. Polymorphisms of the 12 major peroxidases of maize. The 3rd International Conference on Isozymes, April 17-20, 1974, Yale University, New Haven, Connecticut, Academic Press, N. Y.

Brewbaker, J. L. and Y. Hasegawa. 1974b. Nine maize peroxidase loci and their tissue specificities. Maize Genetics Cooperation News Letter 48:35-37.

Brewbaker, J. M., M. D. Upadhya, Y. Makinen and T. Macdonald. 1968. Isoenzyme polymorphism in flowering plants. III. Gel electrophoretic methods and applications. Physiol. Plantarum 21:930-940.

Briggs, W. R., G. Morel, T. A. Steeves, J. M. Sussex and R. H. Wetmore. 1955. Enzymatic Auxin inactivation by extracts of the fern Osmunda cinnamomea L. Plant Physiol. 30:143-148.

Briggs, W. R. and P. M. Ray. 1956. An auxin inactivation system involving tyrosinase. Plant Physiol. 31:165-167.

Brown, B. R. 1967. Biochemical aspects of oxidative coupling of phenols. In: W. I. Taylor and A. R. Battersky eds., Oxidative Coupling of Phenols. pp. 167-201. Marcel Dekker, N. Y.

Burg, S. P. 1962. The physiology of ethylene formation. Ann. Rev. Plant Physiol. 13:265-302.

Chenchin, E. E. and H. Y. Yamamoto. 1973. Distribution and heat inactivation of peroxidase isoenzymes in sweet corn. Jour. Food Sci. 38:40-42.

Chourey, P. S., H. H. Smith and N. C. Combatti. 1973. Effects of x irradiation and indoleacetic acid on specific peroxidase isozymes in pith tissue of a nicotiana amphiploid. Amer. Jour. Bot. 60:853-857.

Christie, G. S. and J. D. Judah. 1954. Intracellular distribution of enzymes. Proc. Roy. Soc. B. 141:420-433.

Cleland, R. and A. C. Olson. 1967. Metabolism of free hydroxyproline in avena coleoptile. Biochemistry 6:32-36.

Conklin, M. E. and H. H. Smith. 1969. Effects of fast neutron versus x—irradiation on development, differentiation and peroxidase isozymes in a genetically tumorous nicotiana amphiploid and its parents. Intl. Jour. Radiat. Biol. 16:311-321.

Darimont, E. and R. Baxter. 1973. Ribosomal and mitochondrial peroxidase isoenzymes of the lentil (Lens culinaris) root. Planta 110:205-212.

Davies, P. J. 1973. Current theories on the mode of action of auxin. Bot. Rev. 39:139-171.

Dawson, C. R. and W. B. Tarpley. 1951. Copper oxidases. In: J. B. Sumner and K. Myrback eds., The Enzymes. 2:454-498. Acad. Press, N. Y.

De Jong, D. W. 1967. An investigation of the role of plant peroxidase in cell wall development by the histochemical method. Jour. Histochem. Cytochem. 15:335-346.

Delincee, H., B. J. Radola and F. Drawert. 1971. Isoelectric properties of gamma-irradiated horse-radish peroxidase. Intl. Jour. Radiat. Biol. 19:93-97.

Denna, D. W. 1974. Genetics and development of the isoperoxidases of Cucurbita peop L. The 3rd International Conference on Isozymes, April 17-20, 1974, Yale University, New Haven, Connecticut, Acad. Press, N. Y.

Dvorak, M. and J. Cernohorska. 1967. Peroxidases of different parts of the pumpkin plant (Cucurbita pepo L.). Biol. Plant. 9:308-316.

Endo, T. 1967. Comparison of the effects of gamma-rays and maleic hydrazide systems of maize seed. Radiat. Bot. 7:35-40.

Endo, T. 1968. Indoleacetate oxidase activity of horseradish and other plant peroxidase isozymes. Plant and Cell Physiol. 9:333-341.

Fahraeus, G. and H. Ljunggren. 1961a. Monophenolase and polyphenolase activity of fungal laccase. Biochim. Biophys. Acta 54:192-194.

Fahraeus, G. and H. Ljunggren. 1961b. Substrate specificity of a purified fungal laccase. Biochem. Biophys. Acta 46:22-32.

Fahraeus, G. and V. Tullander. 1956. Effect of indole-3-acetic acid on the formation of oxisases in fungi. Physiol. Plantarum 9:494-501.

Farkas, G. L. and M. A. Stahmann. 1966. On the nature of changes in peroxidase isoenzymes in bean leaves infected by southern bean mosaic virus. Phytopathology 56:669-677.

Feifl, F. 1966. Spot Tests in Organic Analysis. Elsevier Publishing Co., N. Y.

Fomenko, B. S. 1970. Natural inhibitors and their roles in peroxidase activation in radicles of r-irradiated horse bean sprouts. Radiobiologiya 10:495-498. (Original not seen. Chem. Abstr. 74, 20059g)

Fomenko, B. S. 1971. Action of r-irradiation on the peroxidase and polyphenoloxidase activities of various organs of horse bean. Biol. Nauki. 14:60-64. (Original not seen. Chem. Abstr. 75, 115714b)

Fowler, J. L. and P. W. Morgan. 1972. The relationship of the peroxidative indoleacetic acid oxidase system to in vivo ethylene synthesis in cotton. Plant Physiol. 49:555-559.

Fox, L. R., W. K. Purves and H. I. Nakada. 1965. The role of horseradish peroxidase in indole-3-acetic acid oxidation. Biochemistry 4:2754-2763.

Frenkel, C. 1972. Involvement of peroxidase and indole-3-acetic acid oxidase isozymes from pear, tomato, and blueberry fruit in ripening. Plant Physiol. 49:757-763.

Freudenberg, K., J. M. Harkin, M. Rechert and T. Fukuzumi. 1958. Die an der Verholzung beteiligten Enzyme. Die Dehydrierung des Sinapinalkohols. Chem. Berichte 91:581-590.

Gallagher, P. H. 1924. VII. Mechanism of oxidation in the plant. Part III. Peroxidase. Observations on the thermostability of the peroxidase of the mangold. Biochem. Jour. 18:39-46.

Galston, A. W. and L. Y. Dalberg. 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. Amer. Jour. Bot. 41:373-380.

Galston, A. W. and P. J. Davies. 1970. Control mechanisms in Plant Development. Prentice-Hall, Inc., New Jersey.

Galston, A. W., S. Lavee and B. Z. Siegel. 1968. The induction and repression of peroxidase isozymes by 3-indoleacetic acid. In: F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. pp. 455-472. Runge Press, Ottawa.

Gee, M. S., O. E. Nelson and J. Kuc. Abnormal lignins produced by the brown-midrib mutants of maize II. Comparative studies on normal and brown-midrib-1 dimethylformamide lignins. Arch. Biochem. Biophys. 123:403-408.

Gelehrter, T. D. and G. J. Spencer. 1974. Pseudoisozymes of hepatic tyrosine aminotransferase. The 3rd International Conference on Isozymes, April 17-20, 1974, Yale University, New Haven, Connecticut, Acad. Press, N. Y.

George, P. 1953a. Intermediate compound formation with peroxidase and strong oxidizing agents. Jour. Biol. Chem. 201:413-426.

George, P. 1953b. The chemical nature of the second hydrogen peroxide compound formed by cytochrome c peroxidase and horseradish peroxidase. I. Titration with reducing agents. Biochem. Jour. 54:267-276.

Giacomelli, M. 1967. Peroxidase isozymes in barley leaves from irradiated seeds. Nature 213:1265-1266.

Giacomelli, M. and T. Cervigni. 1964. Changes in peroxidase activity, ascorbic acid and dehydro-ascorbic acid content in Vicia sativa grown under chronic gamma irradiation. Radiat. Bot. 4:395-403.

Goddard, D. R. and H. Stafford. 1954. Localization of enzymes in the cells of higher plants. Ann. Rev. Plant Physiol. 5:115-132.

Goldacre, P. L., A. W. Galston and R. L. Weintraub. 1953. The effect of substituted phenols on the activity of the indoleacetic acid oxidase of peas. Arch. Biochem. Biophys. 43:358-373.

Gordon, J. C. 1971. Changes in total nitrogen, soluble protein, and peroxidases in the expanding leaf zone of eastern cottonwood. Plant Physiol. 47:595-599.

Gordon, A. R. and N. A. Allridge. 1971. Cytochemical localization of peroxidase A in developing stem tissues of extreme dwarf tomato. Canad. Jour. Bot. 49:1487-1496.

Gortner, W. A. and M. Kent. 1953. Indoleacetic acid oxidase and inhibitor in pineapple tissue. Jour. Biol. chem. 204:595-603.

Gregory, R. P. F. and D. S. Bendall. 1966. The purification and some properties of the polyphenol oxidase from tea (Camellia sinensis L.). Biochem. Jour. 101:569-581.

Gray, R. A. 1959. Preparation and properties of 3-indoleacetaldehyde. Arch. Biochem. Biophys. 81:480-488.

Heard, N. F. 1973. Upsurge of particulate peroxidase in ripening banana fruit. Phytochemistry 12:555-560.

Hamill, D. E. 1970. Genetics of peroxidase isoenzyme polymorphisms in maize (Zea mays L.). Ph.D. thesis, University of Hawaii, Department of Horticulture, Honolulu, Hawaii.

Hamill, D. E. and J. L. Brewbaker. 1969. Isoenzyme polymorphism in flowering plants. IV. The peroxidase isoenzymes of maize (Zea mays). Physiol. Plantarum 30:74-78.

Happer, H. A. 1971. Review of Physiological Chemistry. Lange Medical Publication, Calif.

Harborne, J. B. 1964. Biochemistry of Phenolic Compounds. Acad. Press, N. Y.

Hare, R. C. 1964. Indoleacetic acid oxidase. Bot. Rev. 30:129-165.

Harkin, J. M. and J. R. Obst. 1973. Lignification in trees: indication of exclusive peroxidase participation. Science 180:296-298.

Haskins, F. A. 1955. Changes in the activities of several enzymes during germination and seedling development in corn (Zea mays L.). Plant Physiol. 30:74-78.

Haskins, F. A. and H. W. Chapman. 1956. Effects of irradiation maleic hydrazide, temperature, and age on enzyme activity in seedlings of corn (Zea mays L.). Physiol. Plantarum 9:356-362.

Haskins, F. A. and S. C. Downs. 1961. Activities of acid phosphatase, peroxidase and polyphenolase in etiolated shoots from control and irradiated maize seeds. Agronomy Jour. 53:90-93.

Hayward, D. M., W. V. Dashek and R. R. Mills. 1973. Lily pollen peroxidase. Incompatibility Newsletter 2:33-37.

Hepler, P. K., D. E. Fosket and E. H. Newcomb. 1970. Lignification during secondary cell wall formation in coleus: an electron microscopic study. Amer. Jour. Rot. 57:85-96.

Higuchi, T. 1957. Biochemical studies of lignin formation. I. Physiol. Plantarum 10:356-372.

Higuchi, T. 1971. Formation and biological degradation of lignins. Adv. Enzymol. 34:207-283.

Hillman, W. S. and A. W. Galston. 1956. Interaction of manganese and 2,4-dichlorophenol in the enzymatic destruction of indoleacetic acid. Physiol. Plantarum 9:230-235.

Hinman, R. L. and J. Lang. 1965. Peroxidase-catalyzed oxidation of indole-3-acetic acid. Biochemistry 4:144-158.

Holleman, J. 1967. Direct incorporation of hydroxyproline into protein of sycamore cells incubated at growth inhibitory levels of hydroxyproline. Proc. Natl. Acad. Sci. 57:50-54.

Hosoya, T. 1960. Turnip peroxidase. I. Purification and physico-chemical properties of multiple components in turnip peroxidase.

Hoyle, M. C. 1972. Indoleacetic acid oxidase: A dual catalytic enzyme? Plant Physiol. 50:15-18.

Janssen, M. G. H. 1970. Indoleacetic acid oxidase, peroxidase and polyphenoloxidase of pea roots. Acta Bot. Neerl. 19:73-80.

Jensen, W. A. 1955. The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. Plant Physiol. 30:426-432.

Johan, H. E. and J. V. Amin. 1967. The influence of foliar and substrate application of manganese on cotton. Plant and Soil 26:369-379.

Kamerbeek, G. A. 1956. Peroxydase content of dwarf types and giant types of plants. Acta Bot. Neerl. 5:257-263.

Kamisaka, S. and Y. Masuda. 1970. Stimulation of auxin-induced cell expansion in plant tissue by cyclic 3,5-adenosine monophosphate. Naturwiss. 57:546.

Kawashima, N. and I. Uritani. 1965. Some properties of peroxidase produced in sweet potato infected by the black rot fungus. Plant and Cell Physiol. 6:247-265.

Kay, E., L. M. Shannon and J. Y. Lew. 1967. Peroxidase isozymes from horseradish roots. II. Catalytic properties. Jour. Biol. Chem. 242:2470-2473.

Keilin, D. and T. Mann. 1938. Polyphenol oxidase: Purification, nature and properties. Proc. Roy. Soc. London 125B:187-204.

Kenten, R. H. 1955. The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems. Biochem. Jour. 59:110-121.

Kenten, R. H. and P. J. G. Mann. 1953. The oxidation of certain dicarboxylic acids by peroxidase systems in the presence of manganese. Biochem. Jour. 53:498-505.

Khudadatov, A. I., P. Guseinova, G. G. Kasumov and F. Guseinova. 1969. Radiation activation of some oxidation-reduction enzymes in legumes. Eksp. Mutagenez. Rast., Mater. Respub. Nauch. Sess. 145-149. (Original not seen. Chem. Abstr. 77, 14827j)

Kiermeier, F. and F. Herrlinger. 1951. Biologishe Einflusse auf die Hitzeinaktivierung der Peroxydase in Pflanzengeweben. Biochem. Z. 322:106-112.

Klapper, M. H. and D. P. Hackett. 1956. Investigations on the multiple components of commercial horseradish peroxidase. Biochim. Biophys. Acta 96:272-282.

Kondo, K. and Y. Morita. 1952. Phytoperoxidase. II. Isolation and purification of sweet potato peroxidases and their absorption spectra. Bull. Research Inst. Food Sci., Kyto Univ. 10:33-45. (Original not seen. Chem. Abstr. 47, 38931).

Konings, H. 1964. On the indoleacetic acid converting enzyme and its relation to geotropism, straight growth and cell wall properties. Acta Bot. Neerl. 13:566-622.

Ku, H. S., S. F. Yang and H. K. Pratt. 1967. Enzymatic evolution of ethylene from methional by a pea seedling extract. Arch. Biochem. Biophys. 118:756-758.

Ku, H. S., S. F. Yang and H. K. Pratt. 1970. Ethylene production and peroxidase activity during tomato fruit ripening. Plant and Cell Physiol. 11:241-246.

Kuc, J. and O. E. Nelson. 1964. The abnormal lignins produced by the brown-midrib mutants of maize. I. The brown-midrib-1 mutant. Arch. Biochem. Biophys. 105:103-113.

Lanzani, G. A. and E. Galante. 1964. Peroxidase activities from wheat embryo ribosomes. Arch. Biochem. Biophys. 106:20-24.

Larsen, P. 1966. Quantitative determination of indole-3-acetaldehyde. II. Physiol. Plantarum 19:780-784.

Larsen, P. and S. Klungsoyr. 1964. Quantitative determination of indole-3-acetaldehyde. Physiol. Plantarum 17:151-156.

Lavee, S. and A. W. Galston. 1968a. Hormonal control of peroxidase activity in cultured pelargonium pith. Amer. Jour. Bot. 55:890-893.

Lavee, S. and A. W. Galston. 1968b. Structural, physiolgical, and biochemical gradients in tobacco pith tissue. Plant Physiol. 43:1760-1768.

Lechtenberg, V. L., L. D. Muller and L. F. Bauman. 1972. Laboratory and *in vitro* evaluation of inbred and F2 populations of brown midrib mutants of *Zea mays* L. Agronomy Jour. 64:657-660.

Lee, T. T. 1971a. Cytokinin-controlled indoleacetic acid oxidase isoenzymes in tobacco callus cultures. Plant Physiol. 47:181-185.

Lee, T. T. 1971b. Increase of indoleacetic acid oxidase isoenzymes by gibberellic acid in tobacco callus cultures. Canad. Jour. Bot. 49:687-693.

Lee, T. T. 1971C. Promotion of indoleacetic acid oxidase isozymes in tobacco callus cultures by indoleacetic acid. Plant Physiol. 48:56-59.

Lee, T. T. 1972. Interaction of cytokinin, auxin, and gibberellin on peroxidase isoenzymes in tobacco tissue cultured *in vitro*. Canad. Jour. Bot. 50:2471-2477.

Leopold, A. C. 1964. Plant Growth and Development. McGraw-Hill Book Co., N. Y.

Levitt, J. 1972. Responses of Plants to Environmental Stresses. Acad. Press, N. Y.

Lipetz, J. 1962. Calcium and the control of lignification in tissue culture. Amer. Jour. Bot. 49:460-464.

Lipetz, J. and A. J. Garro. 1965. Ionic effects on lignification and peroxidase in tissue cultures. Jour. Cell Biol. 25:109-116.

Liu, E. H. 1971. The use of eugenol and tyrosine as zymogram stains specific for the peroxidase-catalyzed condensation of phenolic compounds. Isozyme Bulletin 4:41.

Liu, E. H. and D. T. A. Lamport. 1968. An hydroxyproline-O-glycosidic linkage in an isolated horseradish peroxidase isozyme. Plant Physiol. 43:S-16.

Liu, E. H. and D. T. A. Lamport. 1973. The pH induced modification of the electrophoretic mobilities of horseradish peroxidase isozymes. Arch. Biochem. Biophys. 158:822-826.

Lodha, M. L., P. C. Mali, A. K. Agarwal and S. L. Mehta. 1974. Changes in soluble protein and isoenzymes in normal and opaque-2 Zea mays endosperm during grain development. Phytochemistry 13:539-542.

Lovrekovich, L., H. Lovrekovich and M. A. Stahmann. 1968. The importance of peroxidase in the wildfire disease. Phytopathology 58:193-198.

Lowry, O. H., N. J. Rosebrough, A. E. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193:265-275.

Lyr, H. 1957. Uber die an der Ligninbildung beteiligten Fermentsysteme. Naturwiss. 44:235.

Mace, M. E. 1967. Indoleacetic acid oxidase activity in banana roots. Canad. Jour. Bot. 45:945-948.

Macko, V., G. R. Honold and M. A. Stahmann. 1967. Soluble proteins and multiple enzyme forms in early growth of wheat. Phytochemistry 6:465-471.

Macnicol, P. K. 1966. Peroxidases of the alaska pea (Pisum sativum L.). Enzymic properties and distribution within the plant. Arch. Biochem. Biophys. 117:347-356.

Macnicol, P. K. 1973. Differential extraction of isoperoxidases from pisum roots. Phytochemistry 12:1269-1272.

Mapson, L. W. and D. A. Wardale. 1968. Biosynthesis of ethylene. Enzymes involved in its formation from methional. Biochem. Jour. 107:433-442.

Margoliash, E. and J. Lustgarten. 1961. The chromatographic forms of cytochrome c. Ann. N. Y. Acad. Sci. 94:731-740.

Markert, C. L. 1968. The molecular basis for isozymes. Ann. N. Y. Acad. Sci. 151:14-40.

Markert, C. L. and F. Moller. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species patterns. Proc. Natl. Acad. Sci. 45:753-763.

Mason, H. S. 1955. Comparative biochemistry of the phenolase complex. Adv. Enzymol. 16:105-184.

Mason, H. S. and M. Cronyn. 1955. On the role of polyphenoloxidase in lignin biosynthesis. Jour. Amer. Chem. Soc. 77:491-492.

McCune, D. C. 1961. Multiple peroxidases in corn. 1961. Ann. N. Y. Acad. Sci. 94:723-730.

McCune, D. C. and A. W. Galston. 1959. Inverse effects of gibberellin on peroxidase activity and growth in dwarf strains of peas and corn. Plant Physiol. 34:416-418.

Meudt, W. J. and T. P. Gaines. 1967. Studies on the oxidation of indole-3-acetic acid by peroxidase enzymes. I. Colorimetric determination of indole-3-acetic acid oxidation products. Plant Physiol. 42:1395-1399.

Milborrow, B. V. ed. 1973. Biosynthesis and Its Control in Plants. Acad. Press, N. Y.

Moore, T. S., Jr. 1973. An extracellular macromolecular complex from the surface of soybean suspension cultures. Plant Physiol. 51:529-536.

Morgan, P. W., H. E. Johan and J. V. Amin. 1966. Effect of manganese toxicity on the indoleacetic acid oxidase system of cotton. Plant Physiol. 41:718-724.

Morita, Y. 1954. Phytoperoxidase. IV. Isolation and purification of broad bean peroxidase. Bull. Research Inst. Food Sci., Kyoto Univ. 15:56-63. (Original not seen. Chem. Abstr. 48, 13752a)

Morita, Y., K. Kameda and M. Mizuno. 1962. Aerobic destruction of indole-3-acetic acid catalyzed by crystalline Japanese radish peroxidase a and c. Agric. Biol. Chem. 26:442-446.

Morita, Y. and K. Kondo. 1954. Phytoperoxidase. III. Isolation and purification of Japanese-radish peroxidase and protein component. Bull. Research Inst. Food Sci., Kyoto Univ. 13:60-66. (Original not seen. Chem. Abstr. 48, 94236b.)

Morris, H. J. 1958. Application of peroxidase test paper in food processing. Food Technol. 12:265-267.

Mudd, J. B., B. G. Johnson, R. H. Burris and K. P. Buchholtz. 1959. Oxidation of indoleacetic acid by quackgrass rhizomes. Plant Physiol. 34:144-148.

Muller, L. D., R. F. Barnes, L. F. Bauman and V. F. Colenbrander. 1971. Variations in lignin and other structural components of brown midrib mutants of maize. Crop Sci. 11:413-415.

Mundt, J. O. and I. E. McCarthy. 1960. Factors affecting the blanching of green beans. Food Technol. 14:309-311.

Neuffer, M. G., L. Jones and M. S. Zuber. 1968. The Mutants of Maize. Crop Sci. Soc. Amer., Wisconsin.

Noltmann, E. A. 1974. The concept of "pseudoisozymes" as multiple enzyme forms different from genetically determined isozymes: Phosphoglucose isomerase from rabbit muscle versus phosphoglucose isomerase from yeast. The 3rd International Conference on Isozymes, April 17-20, 1974, Yale University, New Haven, Connecticut, Acad. Press, N. Y.

Oaks, A. and M. Shaw. 1960. An indoleacetic acid oxidase system in the mycelium of Melampsora lini (Pers.) Lev. Canad. Jour. Bot. 38:761-767.

Ockerse, R., B. Z. Siegel and A. W. Galston. 1966. Hormone-induced repression of a peroxidase isozyme in plant tissue. Science 151:452-453.

Ogawa, M. and I. Uritani. 1970. Effect of gamma radiation on peroxidase development in sweet potato disks. Radiation Res. 41:342-351.

Ogita, Z. 1974. Genetics and biochemical studies on isozymes of hydrolases in serum and urine. The 3rd International Conference on Isozymes, April 17-20, 1974, Yale University, New Haven, Connecticut, Acad. Press, N. Y.

Paul, K. G. 1963. Peroxidases. In: P. D. Boyer, H. Lardy and K. Myrback, eds., The Enzymes. pp. 227-283. Acad. Press, N. Y.

Penon, P. J. P. Cecchini, R. Miassod, J. Ricard, M. Teissere and M. H. Pinna. 1970. Peroxidases associated with lentil root ribosomes. Phytochemistry 9:73-86.

Plesnicar, M., W. A. Bonner, Jr. and B. T. Storey. 1967. Peroxidase associated with higher plant mitochondria. Plant Physiol. 42:366-370.

Poovaiah, B. W. and H. P. Rasmussen. 1973. Peroxidase activity in the abscission zone of bean leaves during abscission. Plant Physiol. 52:263-267.

Price, H. J. Personal communication to author. June, 1972.

Pronin, S. Einfluss der Reaktion des Mediums auf die Regeneration der hiteinaktivierten Peroxydase. Biochem. Z. 236:107-112.

Raa, J. 1973. Cytochemical localization of peroxidase in plant cells. Physiol. Plantarum 28:132-133.

Racusen, D. 1955. Formation of indole-3-aldehyde by indoleacetic oxidase. Arch. Biochem. Biophys. 58:508-509.

Ranadive, A. S. and N. F. Haard. 1972. Peroxidase localization and lignin formation in developing pear fruit. Jour. Food Sci. 37:381-383.

Ray, P. M. 1960. The destruction of indoleacetic acid. III. Relationships between peroxidase action and indoleacetic acid oxidation. Arch. Biochem. Biophys. 87:19-30.

Ray, P. M. and K. V. Thimann. 1956. The destruction of indoleacetic acid. I. Action of an enzyme from Omphalia flavida. Arch. Biochem. Biophys. 64:175-192.

Ridge, I. and D. J. Osborne. 1970. Hydroxyproline and peroxidases in cell walls of Pisum sativum: regulation by ethylene. Jour. Exp. Bot. 21:843-856.

Rychter, A. and S. Lewak. 1971. Apple embryos peroxidases. Phytochemistry 10:2609-2613.

Salomon, D. and J. P. Mascarenhas. 1971a. The effect of cyclic 3,5-adenocine monophosphate on abscission of coleus petioles. Z. Pflanzenphysiol. 65:385-388.

Salomon, D. and J. P. Mascarenhas. 1971b. Auxin induced synthesis of 3,5-adenosine monophosphate in avena coleoptiles. Life Science 10 II:879-885.

Salomon, D. and J. P. Mascarenhas. 1971c. Auxin and cyclic 3,5-adinosine monophosphate during the isolation of chromatin from avena coleoptiles: effects in cell free RNA synthesis. Biochem. Biophys. Res. Comm. 47:134-141.

Sargent, J. A., A. V. Atack and D. J. Osborne. 1974. Auxin and ethylene control of growth in epidermal cells of Pisum sativum: A biphasic response to auxin. Planta 115:213-225.

Sarkanen, K. V. and C. H. Ludwig. 1971. Lignins. Occurrence, Formation, Structure and Reactions. New York, Wiley-Interscience.

Saunders, B. C., A. G. Holmes-Siedle and B. P. Stark. 1964. Peroxidase. The Properties and Uses of a Versatile Enzyme and of some Related Catalysts. Butterworths, Washington.

Scandalios, J. G. 1964. Tissue-specific isozyme variation in maize. Jour. Heredity 55:281-285.

Scandalios, J. G. 1969. Genetic control of multiple molecular forms of enzymes in plants: A review. Biochem. Genetics 3:37-79.

Schwimmer, S. 1944. Regeneration of heat-inactivated peroxidase. Jour. Biol. Chem. 154:487-495.

Seevers, P. M., J. M. Daly and F. F. Catedral. 1971. The role of peroxidase isozymes in resistance to wheat stem rust disease. Plant Physiol. 48:353-360.

Sen, S. P. and A. C. Leopold. 1954. Paper chromatography of plant growth regulators and allied compounds. Physiol. Plantarum 7:98-108.

Sequeira, L. and L. Mineo. 1966. Partial purification and kinetics of indoleacetic acid oxidase from tobacco roots. Plant Physiol. 41:1200-1208.

Shannon, L. M. 1968. Plant isoenzymes. Ann. Rev. Plant Physiol. 19:187-210.

Shannon, L. M., E. Kay and J. Y. Lew. 1966. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. Jour. Biol. Chem. 241:2166-2172.

Shapira, R. and S. Parker. 1960. Artificially induced microheterogeneity in ribonuclease. Biochem. Biophys. Res. Commun. 3:200-204.

Shin, M. and W. Nakamura. 1962. Indoleacetic acid oxidase activity of wheat peroxidase. Jour. Biochem. 52:444-451.

Shriner, R. L. and R. C. Fuson. 1940. The Systematic Identification of Organic Compounds. A Laboratory Manual. John Wiley & Sons, Inc., N. Y.

Siegel, S. M. 1953. On the biosynthesis of lignins. Physiol. Plantarum 6:134-139.

Siegel, S. M. 1954. Studies on the biosynthesis of lignins. Physiol. Plantarum 7:41-50.

Siegel, S. M. 1956. Chemistry and physiology of lignin formation. Quart. Rev. Biol. 31:1-18.

Siegel, S. M. 1957. Non-enzymic macromolecules as matrices in biological synthesis: the role of polysaccharides in peroxidase catalyzed lignin polymer formation from eugenol. Jour. Amer. Chem. Soc. 79:1628-1632.

Siegel, S. M. 1962. The Plant Cell Wall. Macmillan Co., N. Y.

Siegel, S. M. and A. W. Galston. 1955. Peroxidase genesis in plant tissues and its relation to indoleacetic acid destruction. Arch. Biochem. Biophys. 54:102-113.

Sirju, G. and L. A. Wilson. 1974. IAA oxidase preparations from fresh and aged Ipomoea batatas tuber discs. Phytochemistry 13:111-117.

Stafford, H. A. 1965. Factors controlling the synthesis of natural and induced lignins in phleum and elodea. Plant Physiol. 40:844-851.

Stafford, H. A. and S. Bravinder-Bree. 1972. Peroxidase isozymes of first internodes of sorghum. Plant Physiol. 49:950-956.

Staples, R. C., W. J. McCarthy and M. A. Stahmann. 1965. Heat stabilities of acid phosphatases from pinto bean leaves. Science 149:1248-1249.

Stecher, P. G. ed. 1968. The Merk Index. Merck & Co., Inc., N. J.

Steward, F. C. and A. D. Krikorian. 1971. Plants, Chemicals and Growth. Academic Press, N. Y.

Stuber, C. W. and C. S. Levings III. 1969. Auxin induction and repression of peroxidase isozymes in oat (Avena sativa L.). Crop Sci. 9:415-416.

Stutz, R. E. 1957. The indole-3-acetic acid oxidase of Lupinus albus L. Plant Physiol. 32:31-39.

Tagawa, K. and M. Shin. 1959. Hemoproteins of wheat germ. I. Crystallization and properties of peroxidase from wheat germ. Jour. Biochem. 46:865-873.

Tagawa, K., M. Shin and K. Okunuki. 1959. Peroxidases from wheat germ. Nature 183:111.

Tang, Y. W. and J. Bonner. 1947. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. Biophys. 13:11-25.

Taylor, D. M., P. W. Morgan, H. E. Johan and J. V. Amin. 1968. The influence of substrate and tissue manganese on the indoleacetic acid system in cotton. Plant Physiol. 43:243-247.

Theorell, H. 1940. Riversible splitting of a peroxidase. Arkiv. Kemi, Mineral. Geol. 14B, No. 20:1-3.

Thimann, K. V. 1934. Studies on the growth hormone in plants. VI. The distribution of the growth substances in plant tissues. Jour. General Physiol. 18:23-24.

Upadhya, M. D. and J. Yee. 1968. Isoenzyme polymorphism in flowering plants. VII. Isoenzyme variation in tissues of barley seedlings. Phytochemistry 7:937-943.

Van der Mast. 1969. Separation of IAA degrading enzymes from pea roots on columns of polyvinylprolidone. Acta Bot. Neerl. 18:620-626.

Van Fleet, D. S. 1947. The distribution of peroxidase in differentiating tissues. Biodynamica 6:125-140.

Van Fleet, D. S. 1959. Analysis of the histochemical localization of peroxidase related to differentiation of plant tissues. Canad. Jour. Bot. 37:449-458.

Van Overbeek, J. 1935. The growth hormone and the dwarf type of growth in corn. Proc. Natl. Acad. Sci., U. S. 21:292-299.

Wagenknecht, A. C. and R. H. Burris. 1950. Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. Arch. Biochem. Biophys. 25:30-53.

Wang, S. S. and S. Dimarco. 1972. Isolation and characterization of the native, thermally inactivated and regenerated horse radish peroxidase isozymes. Jour. Food Sci. 37:574-578.

Westerfeld, W. W. and C. Lowe. 1942. The oxidation of p-cresol by peroxidase. Jour. Chem. Soc. 145:463-470.

Whitmore, F. W. 1971. Effect of indoleacetic acid and hydroxyproline on isozymes of peroxidase in wheat coleoptiles. Plant Physiol. 47:169-171.

Wilkinson, J. H. 1970. Isoenzymes. J. B. Lippincott Co., Philadelphia.

Yamamoto, H. Y., M. P. Steinberg and A. I. Nelson. 1962. Kinetic studies on the heat inactivation of peroxidase in sweet corn. Jour. Food Sci. 27:113-119.

Yamazaki, I., K. Fujinaga, I. Takehara and H. Takahashi. 1956. Aerobic oxidation of triose reductone by crystalline turnip peroxidase. Jour. Biochem. 43:377-386.

Yamazaki, I. and H. Souzu. 1960. The mechanism of indoleacetic acid oxidase reaction catalyzed by turnip peroxidase. Arch. Biochem. Biophys. 86:294-301.

Yang, S. F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. Arch. Biochem. Biophys. 122:481-487.

Yang, S. F. 1968. Biosynthesis of ethylene. In: F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. pp. 1217-1228. Runge Press, Ottawa.

Yoneda, Y. and T. Endo. 1969. Effect of low concentration of hydrogen peroxide on indoleacetate oxidase zymogram in Pharbitis nil. Plant and Cell Physiol. 10:235-237.

Yonetani, T. and T. Ohnishi. 1966. Cytochrome c peroxidase, a mitochondrial enzyme of yeast. Jour. Biol. Chem. 241:2983-2984.

Yoshida, H. 1883. Chemistry of lacquor (urushi). Jour. Chem. Soc. 43:472-486.